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The role of the inferior olive in the rabbit eyeblink conditioning circuit

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The role of the inferior olive in the rabbit eyeblink conditioning circuit

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LIST OF ABBREVIATIONS

aCSF – artificial cerebrospinal fluid

AP – anterior-posterior

CC – cerebellar cortex

CF – Climbing fibers

CR – conditioned response

CS – conditioned stimulus

DGG – γ-D-glutamylglycine

DV – dorsal-ventral

IN – interposed nucleus

IO – inferior olive

IOD - dorsal accessory inferior olive

IOM - medial inferior olive

IOPr - principal inferior olive

MF – Mossy fibers

ML – medial-lateral

NBQX – 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione

PC – Purkinje cell

SLR – short latency response

Sp5N - spinal trigeminal nucleus

sp5 - spinal trigeminal tract

US – unconditioned stimulus

ABSTRACT

Classical conditioning of the eyeblink response is a form of motor learning that is controlled by the intermediate cerebellum and related brainstem structures. A major component of this neural network is the inferior olive (IO), which is the exclusive source of cerebellar climbing fibers. The functional role of the IO in eyeblink conditioning circuits is not completely understood. Pertinent to eyeblink conditioning, there are two competing concepts explaining IO function. The cerebellar learning hypothesis assumes that, by conveying the unconditioned stimulus (US) input, the IO is supplying the cerebellum with a "teaching" signal required for the acquisition of conditioned responses (CR). This hypothesis predicts that blocking US input should lead to the extinction of CRs. An alternate concept assumes that the IO regulates the tonic activity of the neural network, and predicts that inactivation of the IO would produce dysfunction of the cerebellum. To examine these hypotheses, we designed a series of studies probing the behavioral and electrophysiological effects of blocking US sensory input to the IO. The unique feature of these experiments is that they are the first to document effects of IO manipulations on the neuronal activity in the cerebellum.

In the first series of experiments, we found that blocking glutamatergic sensory inputs to the IO or inactivating the IO gradually abolished CRs. In additional tests we determined that the gradual disappearance of CRs was related to drug diffusion rather than to CR extinction. Since these behavioral effects were paralleled by a dramatic suppression of interposed nucleus (IN) neuronal activity, we concluded that inactivating the rostral parts of the IO complex abolishes CRs by producing a tonic dysfunction of cerebellar eyeblink conditioning circuits.

In the second series of experiments, we tested a recent suggestion that selectively blocking AMPA/kainate receptors in the IO blocks the teaching signal to the cerebellum without affecting the tonic state of the cerebellum. In this pivotal experiment, we found that blocking AMPA/kainate receptors in the IO abolishes CRs by suppressing both the tonic and CR-related neuronal activity in the IN and that the delayed (extinction-like) behavioral effects of NBQX most likely stem from the gradual diffusion of injected drug.

The first two experiments significantly weakened the cerebellar learning hypothesis because they demonstrated that the physiological mechanisms explaining the behavioral effects of common IO manipulations is inconsistent with this notion. We proposed that the ultimate test of the cerebellar learning hypothesis would be blocking IO sensory inputs without upsetting the tonic activity in the cerebellum. To achieve this goal, we initiated a third group of studies to develop a new pharmacological approach that would counter-balance the tonic effects of glutamate blockers. We examined two drug candidates, harmaline and gabazine, for their potential to elevate IO spontaneous activity. In these preliminary studies, we found that the GABA-A antagonist gabazine could recover CRs and IN neuronal activity that had been suppressed by the prior blocking of glutamate receptors with DGG. These ongoing experiments suggest that a near normal level of IO firing is critical for CR performance, and that US signals via the IO are most likely not required for the maintenance of learned responses.

CHAPTER 1. GENERAL INTRODUCTION

1.1 Dissertation Organization

This dissertation investigates the functional role of the inferior olive in a neuronal circuit involved in associative learning. It contains the experimental results obtained by the author during her graduate study under guidance and supervision of her major professor, Dr. Bracha. It is written in an alternative thesis format, containing a general introduction, two research papers, a report of preliminary data for future investigations, general conclusions and acknowledgments.

Chapter 1 includes a short introduction, research hypotheses, background and literature review of the eyeblink classical conditioning paradigm, the neuronal circuit associated with it, description of cerebellar learning hypothesis, inferior olive and the controversy about its functional role in associative learning and memory.

Chapter 2 and Chapter 3 are arranged in a journal format representing two manuscripts in the form prepared for publication:

1. Zbarska S, Holland E, Bloedel JR, Bracha V (2007) Inferior olivary inactivation abolishes conditioned eyeblinks: extinction or cerebellar malfunction? *Behavioral Brain Research*, 178(1): 128-138.

2. Zbarska S, Bloedel JR, Bracha V (2007) Cerebellar dysfunction explains the extinction-like abolition of conditioned eyeblinks following NBQX injections in the inferior olive. Accepted by *The Journal of Neuroscience*.

Chapter 4 contains exploratory preliminary results for future investigation. It is also written in a journal paper format. The closing Chapter 5 contains general conclusions,

recommendations for future research and acknowledgments. References are listed at the end of each chapter.

1.2 Introduction

Associative learning is a fundamental type of memory. One of the best investigated models of associative learning is the classical conditioning of eyeblink reflexes. The learning of classically conditioned eyeblinks critically depends on intermediate cerebellar neural circuits (Thompson, 1990; Yeo, 1991). One of the major components of this neural network is the inferior olive (IO), which is the exclusive source of cerebellar climbing fibers (Desclin, 1974). The role of the IO in this neural circuit is not well understood. There are two major hypotheses explaining the IO's function in eyeblink conditioning. The popular ***cerebellar learning hypothesis*** suggests that the IO provides the cerebellum with an error or "teaching" signal that is essential for learning (Marr, 1969; Thompson, 1986). An alternate concept, termed ***the tonic hypothesis***, assumes that the IO regulates the tonic activity of the system (Strata and Montarolo, 1982; Cerminara and Rawson, 2004). Discriminating between these two hypotheses has been difficult, mostly because of uncertainties associated with IO lesioning/inactivation side-effects and because of methodological difficulties related to long-term neuronal recording during neuropharmacological experiments in animals performing learning tasks. As a result, even though dissociating between these hypotheses requires demonstrating that manipulations of the IO either do or do not alter the tonic activity of the cerebellum, no studies of this kind have yet been performed. In this thesis, we proposed to fill this crucial gap by utilizing our unique method of long term recording of multiple single-units in experiments involving pharmacological manipulations of the IO.

We designed three inter-related experiments that examined predictions of the cerebellar learning and tonic hypotheses both at the cellular and behavioral levels. In the first experiment, we were the first to demonstrate that inactivation of the IO, either with the GABA agonist muscimol or with the wide spectrum glutamate antagonist DGG, abolished learned responses because of cerebellar dysfunction. In the second experiment, we examined whether a more refined block of IO inputs with the more selective AMPA/kainate blocker NBQX could selectively block IO's sensory inputs without disturbing tonic activity in the cerebellum. In this critical experiment, we disproved the previously influential notion that the effects of NBQX on CRs are pivotal evidence for the cerebellar learning hypothesis (Medina et al., 2002). We have shown that tonic effects induced by blocking AMPA/kainate receptors in the IO alter the functional state of involved circuits making the testing of cerebellar learning hypotheses impossible. Results from the first experiment were published while results from the second experiment have been submitted for publication. From this part of the dissertation, we concluded that the tonic hypothesis is true and that the cerebellar learning hypothesis is unlikely but it can not be excluded. We found that future tests of the learning hypothesis will require new tools that would block sensory inputs to the IO without affecting its tonic activity. In our ongoing research, we are examining possible solutions to this problem. Specifically, we are assessing whether tonic effects caused by glutamate blockers can be offset by concurrent application of drugs that enhance IO activity. Here we report highly promising preliminary results from this third experiment.

1.3 Research Hypotheses

The research in this dissertation was based on three inter-related hypotheses of IO function. The first two hypotheses addressed the expected mechanisms of IO inactivation, first at the behavioral and then at the neuronal activity levels. The third hypothesis capitalizes on knowledge we acquired during the first two phases of the research and proposes a principally new approach to addressing IO function.

The first hypothesis focused on the behavioral consequences of IO inactivation, because much of the debate about IO function centered on the timing of the conditioned responses (CR) loss caused by this experimental intervention. Two studies reported that IO inactivation or lesioning abolishes CRs gradually over one (Medina et al., 2002) or several training sessions (McCormick et al., 1985). Since gradual CR abolition resembles behavioral extinction, these findings were viewed as supporting the cerebellar learning hypothesis, which assumes that the IO provides the cerebellum with the unconditioned stimulus information. Observations of gradual CR abolition were contradicted by reports indicating that CR suppression ensues immediately following IO lesioning (Yeo et al., 1986) or inactivation (Welsh and Harvey, 1998). These findings seem to oppose the cerebellar learning hypothesis, as they indicate that IO inactivation induces immediate dysfunction of cerebellar circuits. These contradictory studies were difficult to reconcile because most of them could not exclude whether observed effects were due to blocking non-olivary axons, and because they did not measure the effects of IO manipulations on the cerebellum. To eliminate one of these deficiencies, we inactivated the IO using methods that spare fibers of passage. Specifically, we inactivated the IO with injections of either the glutamate antagonists, DGG or NBQX, or with the GABA-A agonist muscimol. We hypothesized:

Inactivation of the IO by a glutamate antagonist, or inhibition of the IO by a GABA-A agonist, does not lead to CR extinction, but instead produces immediate abolition of learned responses. This will implicate the tonic hypothesis as best explaining IO function.

The second hypothesis addressed neuronal mechanisms of the IO inactivation-related CR suppression. The learning and tonic hypotheses make clear predictions regarding neurophysiological consequences of blocking IO input to the cerebellum. The tonic hypothesis predicts that inactivating the IO should induce tonic dysfunction of cerebellar circuits. On the other hand, the learning hypothesis predicts that blocking the IO should prevent the cerebellum from receiving the information about the unconditioned stimulus, which should result in the gradual disappearance, or extinction, of the neural correlates of CRs. To examine these predictions, it was necessary to measure changes in cerebellar neuronal activity while pharmacologically blocking glutamatergic sensory inputs to the IO. This was an extremely challenging task which we solved using an innovative combination of intracranial microinjections and multi-channel single-unit recordings. In these experiments, sensory inputs to the IO were blocked by one of the glutamate antagonists, DGG or NBQX, and neuronal activity in the cerebellar interposed nuclei was measured with chronically implanted microwire electrode arrays. Since the results of our behavioral experiments supported the validity of the tonic hypothesis, we hypothesized that ***inactivating the IO with a glutamate antagonist suppresses baseline activity in the IN and that this translates into an immediate abolition of CRs. Confirming this will further support the tonic hypothesis of IO function.***

The third hypothesis emerged directly from the outcomes of experiments that tested the first two hypotheses. Results from the first and second series of experiments

demonstrated the validity of the tonic hypothesis of IO function. Paradoxically, showing tonic regulatory effects of the IO did not disprove the learning hypothesis. In principle it is possible that these hypotheses are not mutually exclusive. The IO could regulate cerebellar tonic activity and at the same time it could supply the cerebellum with error signals for learning. If so, then tonic effects elicited by IO inactivation would always obscure processes involved in learning. Could this problem of one physiological process obscuring the other be dissociated experimentally? Theoretically yes, if one successfully blocks sensory inputs in the IO without changing its spontaneous activity. Since sensory inputs in the IO are glutamatergic, blocking these inputs will inevitably decrease IO activity. Revealing a possible teaching function of the IO would then require restoring IO tonic activity by some other means. Here we report exploratory experiments testing if a glutamate block-induced suppression of IO spontaneous activity could be restored or counter-balanced by two drug candidates. The first of them was harmaline, which is an indole alkaloid known to produce rhythmic activation of IO neurons. Our second choice was the GABA-A receptor antagonist, gabazine. These experiments test the following hypothesis: ***Blocking glutamatergic sensory inputs in the IO while preventing major shifts in its spontaneous activity does not abolish CR expression. Therefore, IO-mediated error signals are not essential for eyeblink conditioning.***

1.4 Background and Literature Review

1.4.1 Overview of eyeblink conditioning and its neural circuitry

Eyeblink classical conditioning is a simple form of associative learning. The eyeblink conditioning paradigm is a popular neurobiological model of learning and memory, which has benefited from a detailed knowledge of underlying neural circuits and from exquisite control over the involved learning variables. It has been demonstrated that this form of learning critically depends on intermediate cerebellar neural circuits.

Classical conditioning of the eyeblink reflex

Rabbits, because of their size and docile nature, have become the preferred mammalian model in eyeblink conditioning studies (Gormezano, 1966; Patterson, 1976). Mammals, in general, exhibit an inborn eyeblink reflex (unconditioned response - UR), which can be elicited by aversive stimulation of the eye (Gantt, 1968). In rabbits, eyeblinks consist of the coupled movements of the external eyelids and nictitating membrane. As a type of withdrawal reflex, it can be modified via associative learning to respond to an initially irrelevant conditioned stimulus, which has been paired with the aversive unconditioned stimulus that normally elicits the UR (Gormezano et al., 1962).

The simplest classical conditioning paradigm is delay conditioning, in which the inter-stimulus interval is relatively short and the stimuli co-terminate. In a typical conditioning setup (Fig. 1), rabbits are presented with tone or light stimulation as the conditioned stimulus (CS, Fig. 1 – CS trace), which is paired with the subsequent presentation of a corneal air-puff or peri-orbital shock as the unconditioned stimulus (US,

Fig. 1 – US trace). After repeated paired presentations of the CS and US, rabbits start to respond to the CS with an eyeblink conditioned response (CR, Fig. 1 – CR trace) in anticipation of the upcoming US.

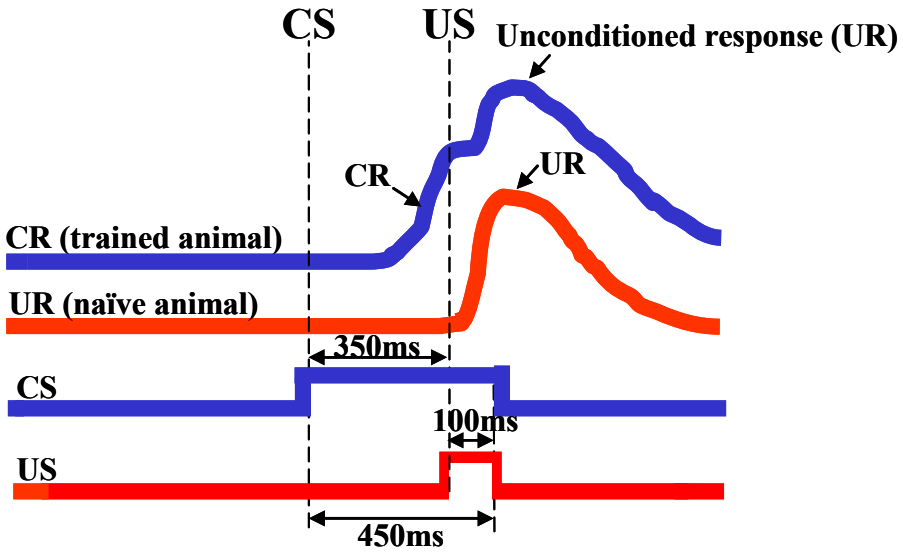

Fig.1. Schematic representation of the delay classical conditioning paradigm.

Circuits subserving classical conditioning of the eyeblink response in the rabbit

Studies conducted during the past three decades have revealed that acquisition and expression of classically conditioned responses is controlled by a neural network consisting of the intermediate cerebellum and several associated brainstem structures (Bracha and Bloedel, 1996; Mauk and Donegan, 1997; Christian and Thompson, 2003; Steinmetz, 2000; Gerwig et al., 2007). To identify the function of individual components of this circuit, investigators used several different techniques, such as lesioning, electrophysiological

recording, electrical stimulation and pharmacological inactivation. It was shown that permanent lesioning or local inactivation of any component illustrated in Fig. 2 prevents acquisition of new CRs and abolishes expression of previously acquired CRs. Furthermore, electrophysiological recordings have demonstrated that activity in these neuronal substrates correlates with the presented stimuli and with CR expression. The inferior olive is a unique structure that responds to the US, and it has a very low frequency of neuronal activity (2-5 Hz, Llinas et al., 2002). The IO's function in eyeblink conditioning was the subject of this thesis.

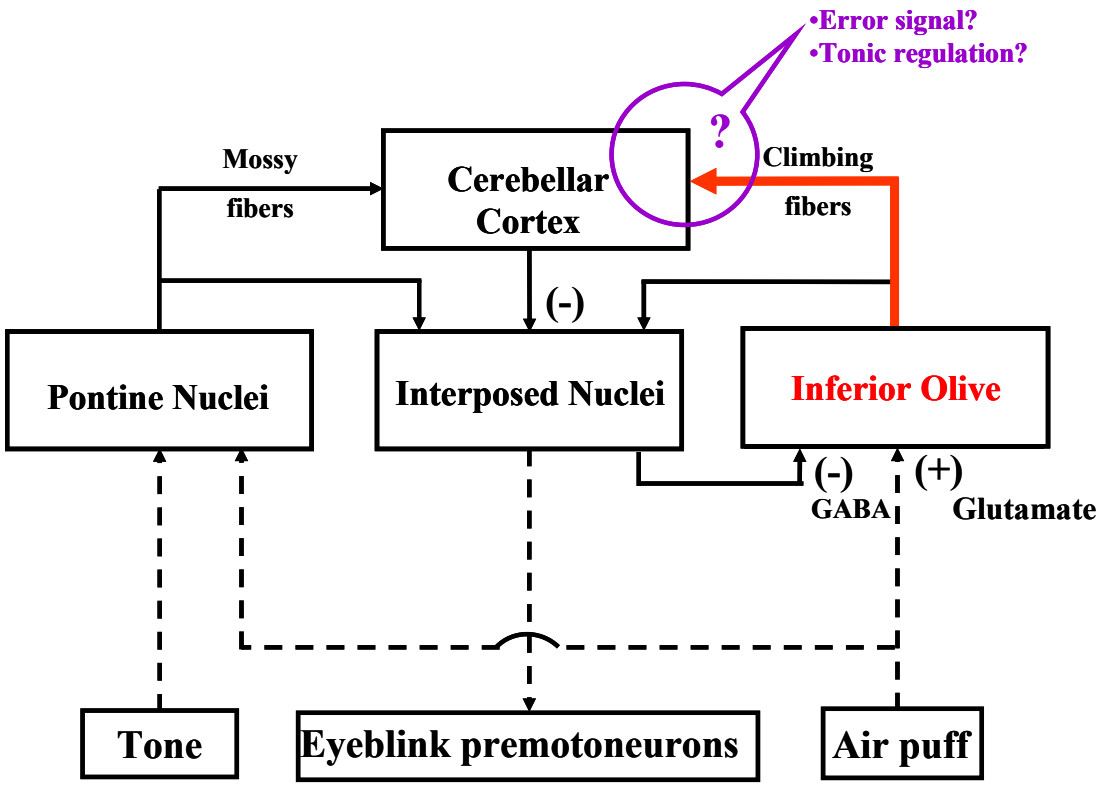

Fig.2. Schematic representation of the cerebellar portion of circuits controlling classical conditioning of eyeblink responses

1.4.2 Cerebellar learning hypothesis

After empirical identification of the brain structures that are essential for eyeblink conditioning (Fig.2), several hypotheses were formulated to explain the operation of this circuit and to propose possible sites of plasticity responsible for CR acquisition and retention. The most commonly discussed in the literature is the cerebellar learning hypothesis. According to this hypothesis, the cerebellar cortex and interposed nuclei are necessary and perhaps sufficient sites of plastic changes for acquisition and retention of discrete CRs in multiple effector systems (e.g., eyeblink, limb flexion). The theoretical basis for this hypothesis of motor learning via synaptic plasticity in the cerebellum is derived from early concepts postulated by Marr (1969) and Albus (1971). It is now assumed that information about the CS arrives to the cerebellum through mossy fibers originating in pontine nuclei (Steinmetz and Sengelaub, 1992). Mossy fibers project to granule and Golgi cells in the granular layer of the cerebellar cortex as well as to the deep nuclei (McCrea et al., 1977). Granule cells send axons to the molecular layer of the cortex where they bifurcate to form parallel fibers. Oriented longitudinally through cerebellar folia, parallel fibers interact with the dendritic field of Purkinje cells, making multiple synaptic contacts. At the same time, information about the US is thought to be conveyed through climbing fibers originating in the inferior olive and projecting both to the molecular layer of the cerebellar cortex, intertwining with Purkinje cells' dendritic field, as well as to the deep nuclei. Thereby, the sensory information from mossy and climbing fibers converges on cortical Purkinje cells and cerebellar nuclear cells (Ito, 1984). According to the cerebellar learning hypothesis, plasticity related to associative learning occurs as a result of this co-activation of mossy and climbing fibers. It is presumed that the heterosynaptic interaction at the points of stimulus

convergence triggers local cellular plastic processes resulting in the changed responsivity of Purkinje and nuclear cells to the CS. These plastic changes result in the network responding to the CS signal by issuing a cerebellar nuclear "motor command" that triggers the CR (Thompson, 1988; Thompson et al., 1997). These command-oriented discharges are expected to strongly correlate with the amplitude-time course of conditioned eyeblinks.

Indeed numerous studies seem to support the cerebellar learning hypothesis. It was shown that inactivating the cerebellar cortex prevents CR acquisition (Hardiman et al., 1996; Attwell et al., 2001) and abolishes CR retention (Gruart and Yeo, 1995) in rabbits. Recently it was found that inactivating the cerebellar cortex right after conditioning disrupts memory consolidation processes and thus prevents learning (Attwell et al., 2002). Likewise, inactivating the IN or blocking protein synthesis in the IN prevents acquisition (Krupa and Thompson, 1997; Bracha et al., 1998) and abolishes CR expression (Chapman et al., 1990; Bracha et al., 1994). Purkinje cells in the cerebellar cortex and neurons in the IN exhibit neuronal activity that precedes and correlates with behavioral CRs (Berthier and Moore, 1990; Aksenov et al., 2004; Aksenov et al., 2005). These findings are consistent with the idea that the cerebellar cortex and IN become responsive to the CS and generate the CR motor command that is sent to eyeblink motoneurons (Thompson, 1986; Green and Steinmetz, 2005).

Although these experiments are consistent with the cerebellar learning hypothesis, they could not exclude involvement of other neuronal structures outside of the cerebellar circuit, e.g., cerebellar efferent targets. It is possible that lesions or pharmacological manipulations of the cerebellum disrupt learning and memory by disrupting plastic changes at extra-cerebellar sites.

1.4.2 Inferior olive and the controversy about its functional role in associative learning and memory

The inferior olive is a unique structure that projects exclusively to the cerebellum, and whose axons are the sole source of a distinct cerebellar input - the climbing fibers (Desclin, 1974). Experimental evidence suggests that the dorsal accessory IO is required for the acquisition and expression of classically conditioned eyeblinks (McCormick et al., 1985; T:urker and Miles, 1986; Yeo et al., 1986; Welsh and Harvey, 1998; Medina et al., 2002; Mintz et al., 1994). Two competing hypotheses - the cerebellar learning hypothesis and the tonic regulation hypothesis - have been advanced to explain the data from the above studies.

Cerebellar learning hypothesis. According to the cerebellar learning hypothesis, the IO input to the cerebellum supplies "teaching" signals, which provide information about the US to learning substrates in the cerebellum. This hypothesis predicts that removal of the IO should prevent CR acquisition. Moreover, removing IO input in trained animals should be equivalent to removing the US, which would lead to the gradual disappearance or extinction of CRs. So, one of the most straightforward tests of the cerebellar learning hypothesis involves eliminating the IO input to the cerebellum while examining the fate of previously learned CRs.

Tonic regulation hypothesis. According to the tonic regulation hypothesis, the function of the IO is to regulate tonic activity in the cerebellum. This hypothesis predicts that inactivation of the IO would disrupt normal intrinsic activity in the cerebellum (Benedetti et al., 1983; Batini et al., 1985), which would prevent CR acquisition in naïve subjects and cause immediate abolition of CRs in trained rabbits (Yeo et al., 1986; Welsh and

Harvey, 1998). Also see section "*Blocking synaptic inputs in the IO and its effects on the cerebellum.*"

Conflicting data

Testing predictions of the above two hypotheses about IO function has proven to be a complex issue. While there is general agreement that rabbits need their IO to acquire and express eyeblink CRs, conflicting reports have emerged on the timing of CR abolition after IO inactivation, which is critical for discriminating between the competing IO hypotheses. McCormick et al. (1985) reported that IO electrolytic lesions abolish CRs gradually during several training sessions. Their findings were supported by Medina et al. (2002), who inactivated the IO with the glutamate receptor antagonist, NBQX, which abolished CRs gradually over one training session. Since gradual CR abolition resembles behavioral extinction, these findings were viewed as supporting the cerebellar learning hypothesis.

Observations of gradual CR abolition have been contradicted by reports indicating that CR suppression ensues immediately following IO electrolytic lesioning (Yeo et al., 1986) or lidocaine inactivation (Welsh and Harvey, 1998). An immediate onset of this CR performance deficit is consistent with earlier studies demonstrating that electrolytic lesioning or cooling of the IO produces dramatic shifts of tonic activity in the cerebellar cortex (Montarolo et al., 1982; Batini et al., 1985; Demer et al., 1985), cerebellar nuclei (Benedetti et al., 1983) and even the red nucleus (Billard et al., 1988). Since normal activity in cerebellar nuclei is required for CR expression (Aksenov et al., 2004), blocking CRs could be a simple consequence of IO inactivation-induced tonic dysfunction of cerebellar circuits.

Determining the precise role of the IO in eyeblink conditioning circuits is pivotal for confirming or disproving the cerebellar learning hypothesis, which is the key to understanding eyeblink conditioning. Technical discrepancies among the differing studies reporting on the timing of CR deficits can only be reconciled by further experimentation. Early electrolytic lesion studies could have inadvertently disrupted axons passing in the IO's vicinity, and may have suffered from a gradual post-lesion degeneration of the IO or its afferent sources. Both of these phenomena could have affected the time course of developing CR deficits. Similarly, inactivating the IO with lidocaine could have blocked passing axons thus confounding the effects specific to IO inactivation. Another weakness inherent to the previous studies was that they did not examine effects of IO manipulations on cerebellar physiology. The most likely reason for this omission was the lack of suitable methods for long-term recording of single-unit activity in awake animals.

The main contribution of the present thesis is that we conducted a systematic series of experiments aimed at surmounting the shortcomings of previous studies. Specifically, we developed pharmacological protocols for blocking specific synaptic inputs to the IO without affecting fibers of passage or the long-term survival of IO neurons. Also, we were the first to perform critical measurements characterizing the shifts in cerebellar single-unit activity induced by IO manipulations in awake rabbits.

Blocking synaptic inputs in the IO and its effects on the cerebellum

The selection of appropriate drugs for blocking the IO sensory inputs and possible interpretations of cerebellar neuronal changes induced by their application are both facilitated by the known aspects of IO physiology.

The IO has a unique anatomical organization. Its neurons are electrotonically coupled, forming gap-junctions (Llinas et al., 1974; Sotelo et al., 1974). The IO has a relatively low frequency of neuronal activity, spontaneously discharging at 2-5 spikes/second (Leznik and Llinas, 2005). The IO consists of several subnuclei that project contralaterally to para-sagittal zones of the cerebellar cortex (Azizi and Woodward, 1987), and they send collaterals to deep cerebellar nuclei that also receive Purkinje-cell projections from the same zone (Kitai et al., 1977; Bernard, 1987; Buisseret-Delmas, 1988; Voogd and Ruigrok, 2004 ;Ruigrok and Voogd, 2000; De Zeeuw et al., 1997). It has been shown that only the rostro-medial part of the dorsal accessory subnucleus is involved in eyeblink classical conditioning (Mauk et al., 1986; Yeo et al., 1986; Sears and Steinmetz, 1991). The climbing fibers from this part of the IO project to Purkinje cells in the HVI zone of the cerebellar cortex, whose inhibitory fibers terminate in the anterior IN (Bishop et al., 1979; Furber and Watson, 1983; Andersson and Hesslow, 1987; De Zeeuw et al., 1997). The IN then sends GABAergic projections to the IO, forming an inhibitory feedback within the olivocerebellar loop (Andersson et al., 1988; Fredette and Mugnaini, 1991; Buisseret-Delmas and Angaut, 1993; Svensson et al., 2005). It has been suggested that the function of cerebellar-olivary inhibition might be to regulate synaptic plasticity resulting from parallel fiber/climbing fiber interaction in cerebellar Purkinje cells, by suppressing the IO after learning has occurred (Hesslow and Ivarsson, 1996; Bengtsson and Hesslow, 2006).

Besides inhibitory inputs, the dorsal accessory IO receives excitatory glutamate afferents transmitting US information from the trigeminal nuclei (Huerta et al., 1985; van Ham and Yeo, 1992). The presence of both GABA- and glutamate-ergic inputs offers two possible approaches to blocking IO input to the cerebellum. One could utilize GABA-A

agonists to inactivate the IO, or glutamate antagonists to block its excitatory sensory inputs. Here we tested both approaches, using the GABA-A agonist, muscimol, and the glutamate antagonists, DGG and NBQX. The feasibility of this approach is supported by previous studies having shown that local injections of GABA and glutamate antagonists alter IO activity and its responses to facial stimulation in anesthetized rats (Lang, 2001; Lang, 2002) and that IO infusions with NBQX abolish CRs in the rabbit (Medina et al., 2002).

1.4.3 Testing the cerebellar learning hypothesis by blocking IO sensory input while maintaining its tonic activity

Experiments described in Chapters 2 and 3 supported the tonic regulation hypothesis of IO function. Does confirming that the IO regulates tonic cerebellar activity disprove the alternate cerebellar learning hypothesis? Not necessarily. The problem is that the two hypotheses do not have to be mutually exclusive. In fact, some investigators have proposed that the IO plays a role both in learning CRs and in regulating cerebellar tonic activity (Bengtsson and Hesslow, 2006). If this proves true, then demonstrating that IO blocking abolishes CRs via cerebellar tonic dysfunction (as shown in Chapters 2 and 3) would not constitute direct evidence against the cerebellar learning hypothesis. This brings us to a fundamental question: Can these two putative functions be dissociated and tested independently?

We hypothesize that the teaching function could be dissociated from the tonic function by independently manipulating the IO's baseline firing that regulates cerebellar tonic activity and the IO's evoked responses to sensory stimulation that were proposed to convey the error signal. Thus, the ideal test of the cerebellar learning hypothesis would

involve examining behavioral and electrophysiological consequences of blocking the US input to the IO without affecting its intrinsic activity. In the ongoing preliminary experiments described in Chapter 4, we have been examining this issue using a combined application of glutamate blockers and two drugs - harmaline and gabazine - that could potentially rescue the IO's spontaneous firing.

Systemic administration of the alkaloid, harmaline, produces synchronized rhythmic activation of IO neurons and induces a strong body tremor (De Montigny and Lamarre, 1975; Llinas and Volkind, 1973). Recordings from the IO during systemic harmaline injections revealed synchronized rhythmic activation in the caudal half of the medial dorsal accessory IO (De Montigny and Lamarre, 1975). The exact mechanism by which harmaline activates the IO has not been identified. The second drug candidate for restoring IO activity is gabazine, a GABA-A receptor antagonist (Ueno et al., 1997). Application of GABA antagonists has been previously shown to increase IO firing rates (Lang, 2002). Effects of these two drugs on the recovery of CRs abolished by a prior injection of the glutamate antagonist DGG were examined in experiments described in Chapter 4.

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CHAPTER 2. INFERIOR OLIVARY INACTIVATION ABOLISHES CONDITIONED EYEBLINKS: EXTINCTION OR CEREBELLAR MALFUNCTION?

A paper published in the journal *"Behavioral brain research"* [1]

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2.1 Abstract

The inferior olive (IO) is a required component of neural circuits controlling the classical conditioning of eyeblink responses. Previous reports indicated that lesioning or inactivating the IO abolishes conditioned eyeblinks (CRs), but there was disagreement regarding timing of the CR performance deficit. As a result, it was not clear whether IO inactivation produces unlearning of CRs or a non-specific dysfunction of cerebellar circuits. Since most of these studies used methods that could block unrelated axons passing through the IO region, additional experiments are required to further elucidate IO function, using inactivating agents that act selectively on cell bodies. In the present study, the IO was inactivated using the glutamate receptor antagonist DGG and the GABA-A receptor agonist muscimol in rabbits performing well-learned CRs. Effects of inactivating the IO on CR expression and on neuronal activity in the anterior cerebellar interposed nucleus (IN) were

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examined. We found that either blocking excitatory glutamate inputs or activating inhibitory GABA inputs to the IO abolished CRs. This effect occurred with variable delay following drug injections. Additional experiments, in which post-injection testing was delayed to allow for drug diffusion, revealed invariably immediate suppression of CRs. This demonstrated that suppressing IO activity using DGG or muscimol does not induce unlearning of CRs. Single-unit recording during DGG injections revealed that CR suppression was paralleled by a dramatic suppression of IN neuronal activity. We concluded that inactivating the rostral parts of the IO complex abolishes CRs by producing a tonic malfunction of cerebellar eyeblink conditioning circuits.

2.2 Introduction

Classical conditioning of the eyeblink response in the rabbit is a popular model for the study of implicit memory. The acquisition and expression of classically conditioned eyeblink responses (CRs) is critically dependent on the intermediate cerebellum and associated circuits. A pivotal component of these circuits is the inferior olive (IO) which is thought to supply the cerebellum with an unconditioned stimulus (US) signal that is required for both the learning and also for the maintenance of already acquired CRs [e.g. 21,30]. However, the precise role of the IO remains unclear, mostly because of discrepancies in reports that examined effects of IO lesioning or inactivation on CR expression.

The inferior olive (IO) is a unique structure that projects exclusively to the cerebellum, and the IO axons are the sole source of a distinct cerebellar input - the climbing fibers. Evidence suggests that the dorsal accessory IO is required for the expression of eyeblink CRs. For example, lesions of the dorsal accessory IO abolish previously established

CRs [30, 19]. Similar to effects of permanent lesions, suppressing IO activity with the sodium channel blocker lidocaine [28], or with the AMPA receptor antagonist NBQX [21], abolished CRs.

While it is agreed that IO inactivation produces abolition of eyeblink CRs, reports on the timing of the CR performance deficit are conflicting. Two studies reported that IO inactivation or lesioning abolishes CRs gradually over one [21] or several training sessions [19]. Since gradual CR abolition resembled behavioral extinction, these findings were viewed as supporting the cerebellar learning hypothesis, which assumes that the IO provides the cerebellum with the unconditioned stimulus (US) information. According to this concept, blocking the IO prevents the US signal from reaching cerebellar substrates required for learning, thus leading to CR extinction.

Observations of gradual CR abolition were contradicted by reports indicating that CR suppression ensues immediately following IO lesioning [30] or inactivation [28]. An immediate onset of this CR performance deficit is consistent with earlier suggestions that the IO regulates tonic activity in the cerebellum [2, 22]. These authors reported that inactivating the IO increases tonic activity of cerebellar cortical Purkinje cells, thereby suppressing neuronal activity in deep cerebellar nuclei. Since normal activity in cerebellar nuclei is required for CR expression [1], blocking CRs could be a simple consequence of IO inactivation-induced tonic dysfunction of cerebellar circuits.

Due to these discrepancies in experimental findings, the precise role the IO plays in CR control remains unclear. The differing reports on the timing of CR performance deficits are associated with divergent interpretations of IO function and are difficult to reconcile without further experimentation. Early electrolytic lesion studies could have been affected

by inadvertent disruption of axons passing in the IO vicinity or by a gradual post-lesion degeneration of the IO or its afferent sources. Both of these phenomena could have affected the time course of developing CR deficits. Similarly, inactivating the IO with lidocaine could have blocked passing axons thus confounding effects specific to IO inactivation.

To resolve these issues, we designed experiments whose objective was to reversibly inactivate the IO without affecting fibers of passage in rabbits producing well-learned CRs. The unique feature of these experiments is that they combine, for the first time, selective pharmacological manipulation of the IO with simultaneous behavioral and electrophysiological analysis. Since understanding the behavioral effects of IO manipulations depends on knowing the impact of these treatments on cerebellar activity, we combined the behavioral measurements of CRs with electrophysiological recordings of cerebellar nuclear activity. We hypothesized that suppressing neuronal activity in the contralateral dorsal accessory IO abolishes CRs due to a tonic malfunction of cerebellar eyeblink conditioning circuits.

It is known that the IO receives inhibitory GABA and excitatory glutamate afferents and those local injections of GABA and glutamate receptor antagonists alter IO activity in anesthetized rats [16, 17]. Thus, either GABA receptor agonists or glutamate receptor antagonists could be used to suppress IO activity. Here we report effects of microinjecting the GABA-A receptor agonist, muscimol, and the glutamate AMPA/kainate and NMDA receptor antagonist, DGG, in the IO of rabbits exhibiting well-learned CRs. We found that both drugs abolished CRs with a variably delayed effect. Introducing a short waiting period to allow for drug diffusion following drug injections produced an immediate abolition of CRs. Exploratory recordings of neuronal activity in cerebellar interposed nuclei (IN)

demonstrated that blocking glutamate neurotransmission in the IO leads to decreased IN neuronal firing rates. These results indicate that inactivating the dorsal accessory IO abolishes CRs via the tonic dysfunction of neuronal activity in the intermediate cerebellum.

This paper has been previously presented in abstract form [31].

2.3 Materials and Methods

2.3.1 Subjects

Eleven male, New Zealand White Rabbits (Harlan, Indianapolis, Indiana), weighing 2.5-3.0 kg (3-4 months old at the beginning of experiments), were used in these studies. Eight rabbits were included in a study examining effects of IO inactivation on CR expression (IO injection group). The remaining three animals were used in experiments testing effects of IO inactivation on CRs and on neuronal activity in the IN (IN recording group). Rabbits were housed individually on a 12/12 hr light/dark cycle and provided with food and water ad libitum. All experiments were performed in accordance with the National Institutes of Health's "Principles of Laboratory Animal Care" (publication No. 86-23, revised 1985), the American Physiological Society's "Guiding Principles in the Care and Use of Animals," and the protocol approved by Iowa State University's Committee on Animal Care.

2.3.2 Surgery

Surgical implantation of injection and recording hardware was performed on naïve animals using sterile surgical techniques. Rabbits were anesthetized with a mixture of ketamine (50 mg/kg), xylazine (6 mg/kg), and acepromazine (1.5 mg/kg) and placed in a stereotaxic apparatus with lambda positioned 1.5 mm below bregma. After exposing the

skull, three stainless steel anchor screws and a 28-gauge injection guide tube were implanted. For targeting anterior parts of the right IO, the following stereotaxic coordinates (relative to lambda) were used: AP=x-(0.69x+4.5) mm (x is horizontal distance between bregma and lambda in mm), ML=1.0 mm and DV=22.4 mm. The patency of the injection guide tube between experiments was protected by a 33-gauge stainless steel stylet.

The three rabbits in the IN recording group were also implanted with a microelectrode array/micromanipulator assembly targeting the left IN as in [1]. The manipulator contained three bundles of microwire electrodes totaling 14 electrodes (stainless steel, 18 μm diameter, Formvar insulation). The microwires were connected to a 15-pin connector embedded in dental acrylic. The stereotaxic coordinates for the IN implant were: AP=x-(0.69x+4.8) anterior to lambda (x is distance between bregma and lambda in mm); ML=5.0 mm lateral to lambda, and DV=14 mm ventral to lambda. The guide tube, manipulator, connector, anchor screws, and a small Delrin block designed to accommodate an air-puff delivery nozzle and an eyeblink sensor were secured in place with dental acrylic. Following surgery, the manipulator and injection guide tube were protected with removable Delrin caps. All animals were treated with antibiotics during a 5-day post-surgical recovery period.

2.3.3 Training procedures

Before any training began, all rabbits were first adapted to a restraint box in a sound-attenuated experiment chamber for 3 days, 30 min per day. Head movements were not restricted either during adaptation or during experiments. Adapted animals were conditioned in the standard delay classical conditioning paradigm until they reached 90% of conditioned

responses (CRs) for at least two consecutive days. The conditioned stimulus (CS) was a binaural 450-ms, 80-dB SPL, 1-kHz tone, superimposed on a 70-dB white noise. As unconditioned stimulus (US), a 100-ms, 50-psi (at the source) air puff was applied to the left eye. The inter-stimulus interval was 350 ms. The inter-trial interval varied from 15 to 25 s pseudo-randomly. Each rabbit had one conditioning session of 100 paired trials per day.

2.3.4 Injection procedures

Before each injection experiment a 33-gauge stainless steel needle was inserted in the guide tube. The injection needle was connected to a 10-µl Hamilton syringe using transparent Tygon tubing. The intracranial microinjections were performed manually at a rate of 0.5 µl/min. The injected volume was monitored by observing the movement of a small air bubble relative to gradation marks on the transparent tubing. During injection sessions, 40 trials of paired stimuli were applied prior to the injection to assure that needle insertion had no effect on CR performance. Drugs were not injected if rabbits had less than 85% CRs during the pre-injection baseline period.

Injection experiments were conducted in two stages. The first was the functional mapping stage when the effective depth for injections was determined. In the second stage of injection experiments, effects of drugs on CR performance were measured and compared. Since previous studies have shown that AMPA receptor blockers decrease neuronal activity in the IO [17] and abolish eyeblink CRs [21], we used 1.0-µl injections of the AMPA/kainate and NMDA receptor blocker, DGG (Tocris Bioscience, 100 nmol in 1µl), for functional mapping. During the mapping stage, the first exploratory DGG injection was performed at the depth of the guide tube tip. If no effects on CR incidence were observed, the animal was

injected 0.5 mm deeper the next day. This daily increase of injection depth was repeated until the DGG injection completely abolished CRs.

Once the effective injection site was determined, the second stage of the experiment ensued. Sessions at this stage consisted of 40 pre-injection trials and 120 post-injection trials, which were administered without delay immediately following the injection. Since CRs were abolished gradually in most rabbits, all animals were tested in additional sessions that included a waiting interval following the injection and before resuming CS-US stimulation trials. The purpose of this waiting interval was to allow for effective drug diffusion before trials resumed. The duration of this waiting interval in each rabbit was equal to the time it took to abolish CRs in its previous no-waiting session. In the three rabbits from the IN recording group, the post-injection period lasted either until the drug effect recovered, or until 260 trials.

On days following successful DGG injections, rabbits were injected either with 1.76 nmol in 0.5 µl of the GABA-A receptor agonist, muscimol (MP Biomedicals (formerly ICN), or with a control injection of the corresponding volume of vehicle. In two rabbits with the most rostral placement of injection needles, the volume of muscimol was increased to 1 µl because the original drug amount did not suffice to completely abolish CRs. In addition, effects of all drugs were measured in waiting period experiments (see above). All drugs were dissolved in artificial cerebrospinal fluid (aCSF, [21]) and their pH was adjusted to 7.4 ± 0.1. Only one drug was injected on any given experiment day.

2.3.5 Data recording and analysis

In the IO injection group, the eyeblink was recorded using an electro-mechanical lever system coupled to the upper eyelid before each session [7]. In rabbits from the IN recording group, eyeblinks were measured using a new, wide field-of-view infrared sensor [23]. The output of sensors was amplified, digitized at 1 kHz with 12-bit resolution, and displayed and stored on a custom data acquisition system. Data were acquired for 1400 ms in each trial, starting 250 ms before CS onset. Eyeblink responses in the time frame between 80 ms after CS onset and US onset were considered CRs when they exceeded the mean of the signal in the 250-ms baseline period by more than five standard deviations (approximately 0.1 mm). This relatively low threshold was selected to capture small responses that could be present during an incomplete drug effect or when the drug effect starts to recover. Spontaneous responses were defined as trials in which the difference between the maximum and minimum values in the baseline period exceeded 0.5 mm. Any blink exceeding the CR threshold within 80 ms after CS onset was recognized as an alpha response. Trials containing spontaneous blinks or rare alpha responses were stored but were not included in further data analyses. Measurements of eyeblink responses were used for calculations of CR incidence per block of 10 trials in each session.

In addition to the visualization of eyeblink traces on the computer monitor, two video cameras were installed in the experiment chamber to monitor general animal behavior during experiments. One camera provided a front view of the rabbit's head and the other one was positioned on the side of the trained eye. This setup proved to be invaluable for detecting drug injection side effects.

In the IN recording group, multiple single-unit signals from microwire electrodes were fed through a custom miniature 14-channel FET-based preamplifier to a multi-channel differential amplifier system (Grass-Telefactor model 12 Neurodata System). The amplified and bandpass-filtered (300 Hz-3 kHz) signal was digitized (25 kHz/channel) using a custom data acquisition system, and was displayed and stored in 1400-ms epochs corresponding to individual trials. Unit discrimination was performed offline using threshold detection followed by a cluster analysis of scatter plots of time and amplitude distances between the peak and valley of individual action potential waveforms. The discriminated data were processed using custom software in addition to a commercial data analysis package (NeuroExplorer, Nex Technologies). Cross-correlation analysis was performed for each unit to eliminate multiple inclusions of the same unit recorded on different wires within a bundle. Raster and peri-event histograms were constructed for each unit. Separate histograms were constructed for baseline trials, trials after the injection, and trials at the end of session representing recovery. In each histogram, the baseline firing rate (250 ms before CS onset) and the timing of significant excitatory and inhibitory changes were computed.

Baseline means of individual cell histograms were pooled together within DGG and control experiments and statistically analyzed. Only units that were isolated reliably throughout the experiment, as judged by the invariance of the shape and size of action potentials, were included in the data analysis.

For the statistical analysis, we used repeated measures ANOVA with the following factors: drug (3 levels: drug, drug with delay, and aCSF) and time/dose (3 levels: before injection, after injection, and recovery). The repeated factor was the four 10-trial blocks that comprise each phase of an experiment. For the analysis of IN group cell activity, the

ANOVA included an additional blocking factor (rabbit) to account for multiple cells recorded from the same animal. We generated planned contrasts from the ANOVAs to address hypotheses regarding patterns of CR incidence and baseline activity of IN neurons arising from the interaction between treatments and phases of experiment. Specifically, using groups of 10-trial blocks, we performed 1-df contrasts between pre-injection, post-injection and recovery within and between experiments with no time delay, with time delay, and their controls. All group data are reported as mean ± standard error of the mean. All statistical analyses were performed using Statsoft Statistica software.

2.3.6 Histology

After all experiments were completed, rabbits were deeply anesthetized with a mixture of ketamine (50 mg/kg), xylazine (6 mg/kg), and acepromazine (1.5 mg/kg). The injection site was marked by injecting 1 µl of tissue-marking dye. Anesthetized animals were transcardially perfused with 1 L of phosphate-buffered saline followed by 1 L of fixative (10% buffered formalin) and 1 L 10% potassium ferrocyanide in 10% formalin. The potassium ferrocyanide was applied only to IN-recording rabbits, where the location of electrodes was marked by passing 10 µA anodal DC current through each wire for 20 s. Whole brains were removed and placed in a solution of 30% sucrose and 10% formalin. One week later, brains were sectioned coronally at 50 µm on a freezing microtome. Sections were mounted onto gelatin-coated slides, dried, and stained with either Luxol blue and neutral red (IO injection group) or with ferrocyanide hydrochloride and neutral red (IN recording group). Locations of the injection sites were determined using bright field microscopy and transferred to a standardized set of coronal sections of the rabbit medulla.

Locations of electrode marks were transferred to a standardized set of coronal sections of the rabbit cerebellar nuclear region.

2.4 Results

2.4.1 Location of injection sites and general observations

The histological reconstruction of injection sites in the IO injection group revealed that they were located in the close vicinity of the rostral portion of the inferior olivary complex (Fig.1). The ranking of injection sites based on the rate of the drug-induced CR abolition indicated that the most effective injection sites were just dorsal or within the dorsal accessory olive; rostro-caudally, these sites were also closest to the most rostral pole of the medial accessory olive (Fig. 1 D and E).

All rabbits in the IO injection group reached the 90% CR criterion in 6 to 18 sessions. The mean CR incidence was 91.4±1.7% and 91.1±0.6%, respectively, in the last two training sessions. Once effective injection sites were established, microinjections of both DGG and muscimol reliably abolished CRs, albeit with a variable delay (see sections 3.2 and 3.3 for additional information). Besides the suppression of CRs, additional side-effects of both drugs were observed. In general, at the given drug concentrations and volumes, muscimol yielded stronger behavioral effects than did DGG. Thus, to minimize the spatial extent of inactivation, a smaller volume of muscimol (0.5 μl) was used when sufficient to abolish CRs. In most experiments, increased tonic eyelid aperture was detected following muscimol and DGG injections. Two subjects with the most laterally placed injection sites exhibited bilateral nystagmus, head rotation to the right, and a postural asymmetry characterized by limb extension on the right side of the body once released from the restraint box. Four

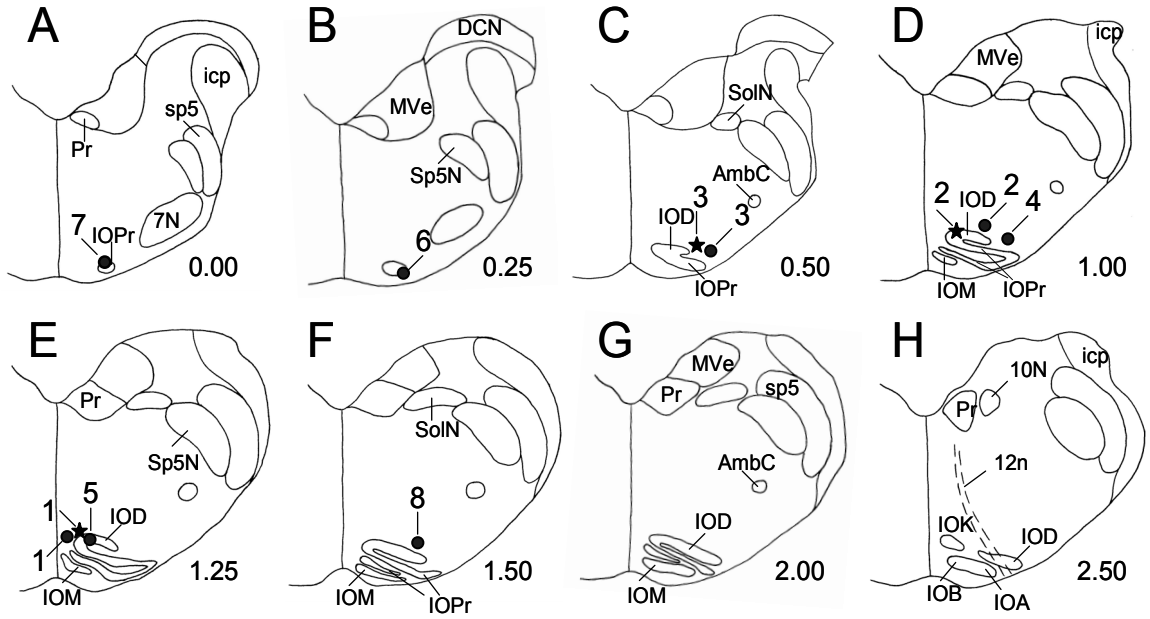


Fig. 1. Location of injection sites in the IO injection group (●, n=8) and in the IN recording group (★, n=3). The identified sites in individual animals were transferred to a set of standardized coronal sections of the rabbit medulla, arranged in rostral-to-caudal order (*A-H*). All injection sites were found within or close to the rostral part of the inferior olivary complex. Injections of DGG at all marked sites abolished CR expression. The animals were ranked based on the aggregate rate of the DGG- and muscimol-induced CR abolition (fastest effect was ranked 1), and their rank is indicated by the number associated with each injection site mark. The anterior-posterior distance of each section from the most rostral pole of the IO in mm is labeled on the lower right side of individual sections. IOD - dorsal accessory inferior olive; IOM - medial inferior olive; IOPr - principal inferior olive; IOA - subnucleus A of the medial inferior olive; IOB - subnucleus B of the medial inferior olive; IOK - cap of Kooy of the medial inferior olive; Sp5N - spinal trigeminal nucleus; sp5 - spinal trigeminal tract; Pr - prepositus hypoglossal nucleus; 7N - facial nucleus; icp - inferior cerebellar peduncle; MVe - medial vestibular nucleus; SolN - solitary nucleus; AmbC - ambiguous nucleus; 12n - hypoglossal nerve; 10N - dorsal motor nucleus of the vagus; DCN – dorsal cochlear nucleus.

rabbits exhibited short periods of sneezing after drug injections. While the timing of eyelid opening usually paralleled effects on CRs, other behavioral effects such as nystagmus and head rotation developed later during the experiment. All of these effects were more pronounced following muscimol injections, indicating that this drug inactivated a larger area of the brain or produced more complete local inactivation.

2.4.2 Effect of DGG on CR expression

Immediate post-injection test. Injecting the AMPA/kainate and NMDA receptor blocker, DGG, at effective injection sites abolished CR expression. Typically, rabbits exhibited high CR incidence during the 40 trials before injection, then during post-injection trials CRs gradually disappeared (Fig. 2 A); in the example illustrated in Fig. 2 A, the effect did not recover during the 80 post-injection trials. Injections of the vehicle at the same site had no effect on CR incidence (Fig. 2 C).

The onset of CR abolition varied between rabbits from an immediate effect to approximately 15 min after the drug injection. Recovery of CRs at the end of an injection session was observed in three out of eight rabbits. Pooled data from eight IO injection animals revealed that CR incidence gradually decreased from 87.5±6.2% in the first post-injection block of 10 trials to 2.5±1.6% in the fifth post-injection block (Fig.3 A). During the last block of trials, CR incidence was 7.5±4.1% (Fig.3 A). This decline of CR frequency contrasted with stable CR performance in the control experiment before and after vehicle (Fig. 3 A). The repeated measures ANOVA yielded significant drug effects. While the planned comparisons revealed a significant effect of DGG ($F_{1,63}$=110.668, $p<0.05$) when

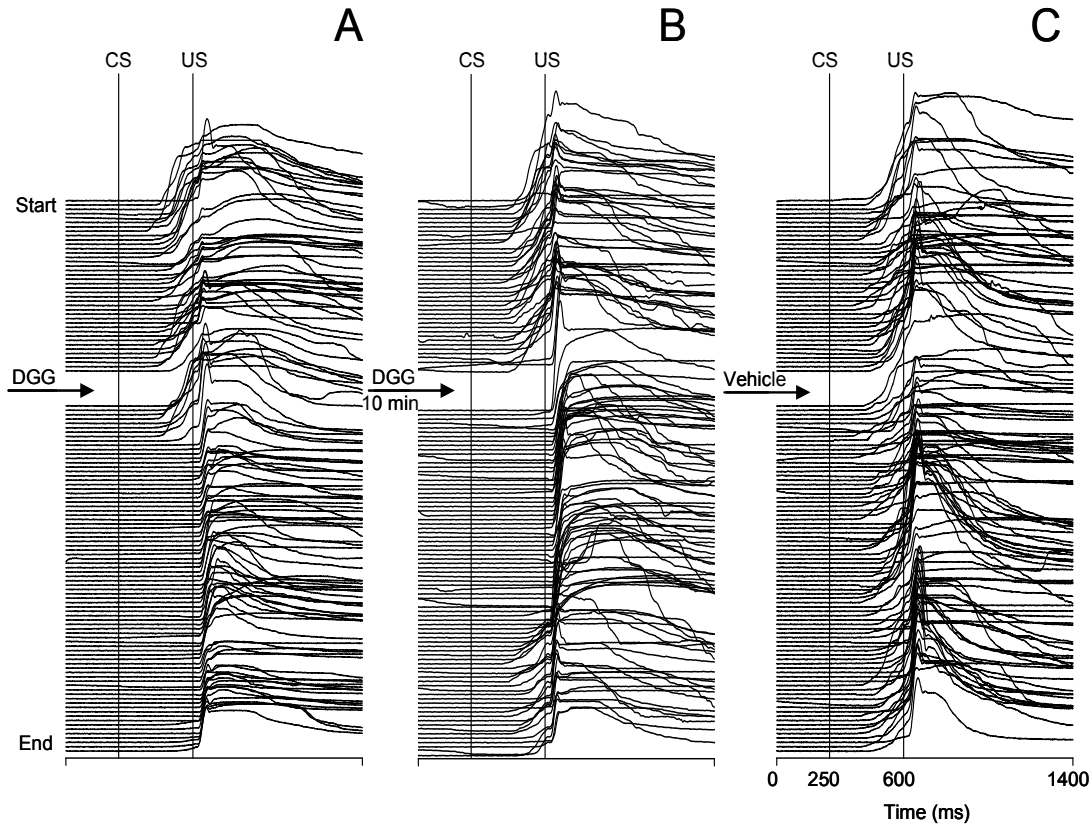


Fig. 2. Individual examples of behavioral effects of microinjecting DGG and vehicle (aCSF) into the IO of the same subject. Each stack plot represents a complete printout of eyeblinks from a 120-trial injection experiment. The experiments start at the top, and each trace represents one trial. Drug injections are denoted by an arrow in the each stack plot. Conditioned eyeblinks are upward deflections of the signal between the CS and US onset markers. *A*: Eyeblinks in an experiment where CR testing was resumed immediately following the injection of 1.0 µl of DGG. Note that CRs were gradually abolished shortly following the DGG injection. *B*: Stack plot of eyeblink mechanograms in an experiment in which the eyeblink CR testing was delayed by inserting a waiting period for DGG diffusion. In this test, CRs were absent immediately after stimuli resumed, indicating that the delayed drug effect in part A was due to drug diffusion. Note the partial recovery of CRs at the end of the waiting experiment. *C*: Eyeblinks in the control experiment in which the injection of 1.0 µl of vehicle (aCSF) did not affect CRs.

comparing pre-injection performance with CR incidence in all post-injection blocks, effects of vehicle were insignificant.

The waiting period test. The waiting period test was administered to examine the role of post-injection stimulation in the gradual abolition of CRs following DGG injections. It was expected that if gradual CR abolition in the previous non-waiting test represented CR extinction, then postponing the CS-US presentation following the DGG injection should have no effect on the timing of CR abolition. This prediction was not confirmed. Fig. 2 B illustrates that when a waiting period was inserted between the drug injection and post-injection testing, CRs were absent immediately from the beginning of the post-injection test. In most waiting experiments, CRs partially recovered at the end of post-injection testing (Fig. 2 B).

At the group level, CR incidence precipitously declined from 84.0±3.3% in the last block of pre-injection trials to 1.3±1.3% during the first 10 post-injection trials (Fig. 3 A). Related to the extended period of observation after injections with wait periods, CRs tended to recover partially toward the end of these experiments, starting from the fourth post-injection block of trials. This recovery was observed in three out of eight rabbits with one of them illustrated in Fig. 2 B. In the last 10-trial block of the waiting period test, mean CR incidence in the IO injection group was 25.0±13.6%. The post-injection decline in CR incidence was significant both when compared to the pre-injection period ($F_{1,63}$=128.580, $p<0.05$) and to the corresponding block of 40 post-injection trials in the non-waiting test ($F_{1,63}$=12.086, $p<0.05$). During the last 40 trials of the waiting period test CR incidence had partially recovered to 22.8±6.2%, which was significantly different from the corresponding block of trials in the non-waiting test (6.6±2.5 %, $F_{1,63}$=4.523, $p<0.05$).

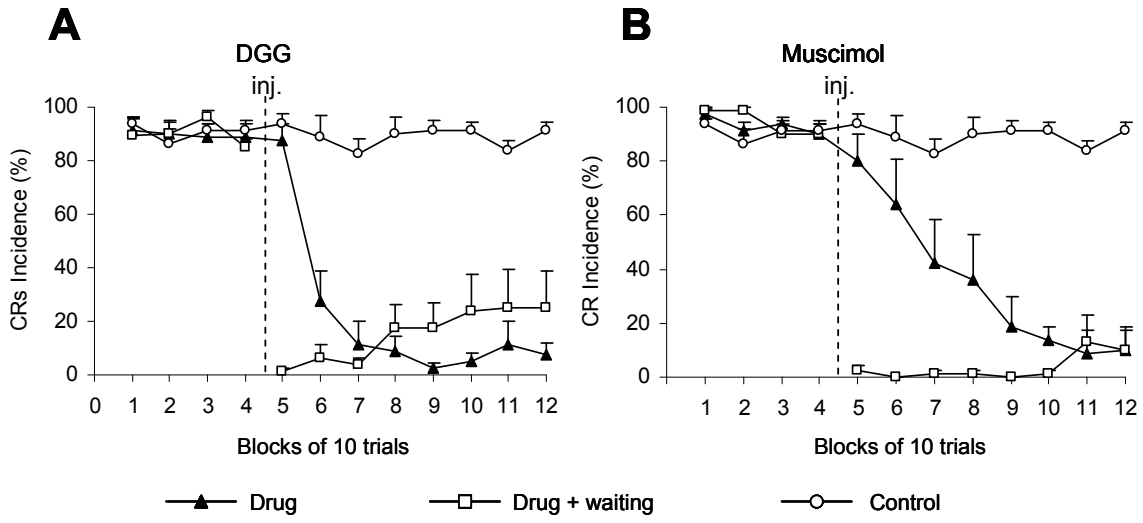


Fig. 3. Effects of DGG, muscimol, and vehicle on CR incidence (±SE) in the group of IO injection rabbits (n=8). A: Effects of DGG when tested immediately following the injection (▲) and following the diffusion waiting period (□) are compared to those observed following the control injection of vehicle (○). Injections (inj.) were administered between the fourth and fifth blocks of trials and are denoted by a vertical dashed line. Note that up to 4 blocks of trials were required to obtain complete CR abolition in the non-waiting test, while CRs were immediately absent in the first post-injection block of the waiting test. B: Effects of muscimol injections in the same group of animals as in A. When the effect of 0.5 µl - 1.0 µl of muscimol was tested immediately following the injection (▲), CR incidence gradually declined until the end of testing. When the resumption of post-injection CS+US trials was delayed until after a pre-specified diffusion waiting period, CRs were immediately abolished (□). Injection of vehicle (aCSF) did not affect CR incidence (○).

2.4.3 Effect of muscimol on CRs

Immediate post-injection testing. To examine effects of suppressing IO activity via activating local GABA receptors, microinjections of the GABA-A receptor agonist, muscimol, were administered at the same injection sites as DGG. Similar to DGG, injections of muscimol gradually suppressed CRs (Fig.4 A). Typically, the effect of muscimol was long-lasting, as the CR deficit recovered in only one rabbit (Figs. 4 A-B). The onset of the muscimol-induced CR suppression appeared to be slower than that following DGG injections. This slower effect relative to DGG could have been related to the smaller volume of muscimol injections, to a possible difference in diffusion rate, or to a more developed gliotic scar at the time of muscimol injections. The onset of muscimol's action varied among rabbits from an immediate effect to 20 min following the muscimol injection. Across all animals, CR incidence gradually decreased from 80.0±10.4% in the first 10 post-injection trials to 8.75±8.75% in the seventh post-injection block (Fig. 3 B). The repeated measures ANOVA indicated significant effects of drug and time. CR incidence in all post-injection trials of the muscimol test was significantly smaller than the pre-injection level ($F_{1,63}$=88.860, $p<0.05$) and the corresponding post-injection block of trials in the control experiment ($F_{1,63}$=115.7868, $p<0.05$).

The waiting period test. Inserting a waiting period following muscimol injections to allow for drug diffusion had a clear effect on the timing of CR suppression (Fig. 4 B). Following the waiting period, CRs were absent immediately and remained abolished across all rabbits (Fig. 3 B). Incidence of CRs dropped from 94.3±1.8% pre-injection to 2.5±1.6% immediately post-injection (Fig.3 B), and CRs remained essentially absent throughout the

first 40 post-injection trials (F1,63=212.902, p<0.05). This immediate reduction of CR incidence contrasts with gradual abolition of CRs in the non-waiting test (F1,63=56.979, p<0.05). Control injections of vehicle had no effects on CR expression (Figs. 3 B, 4 C).

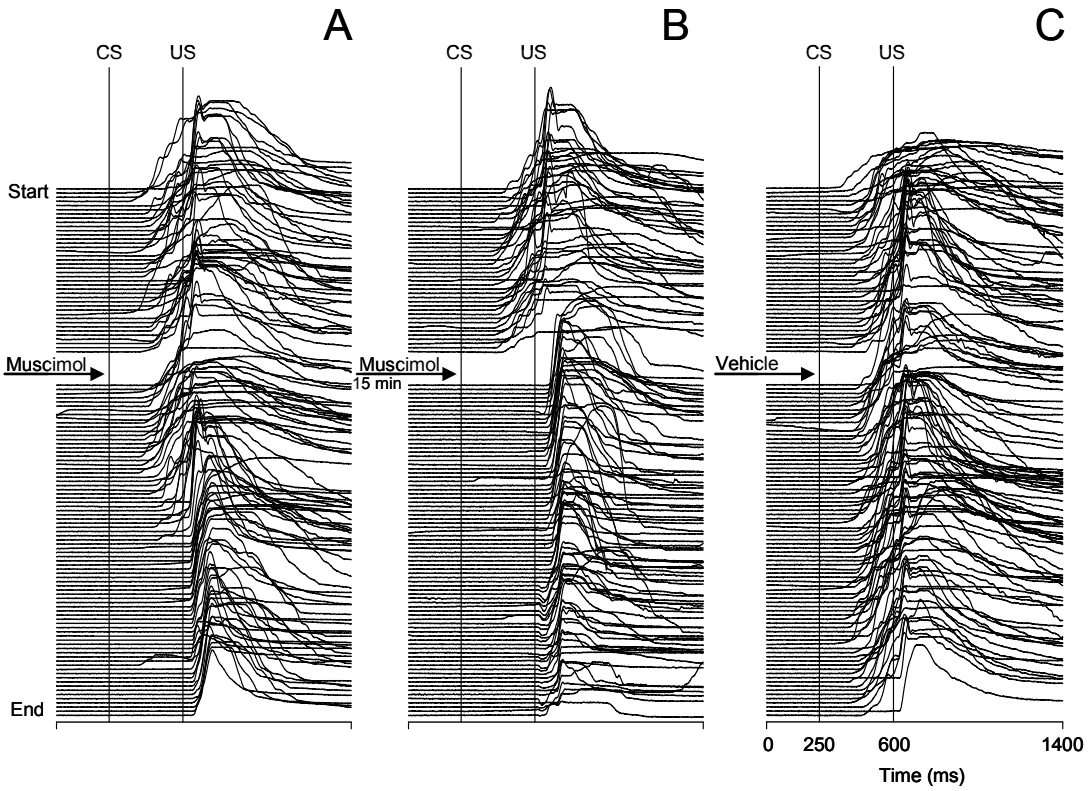


Fig. 4. Individual example of muscimol-induced suppression of CRs. Stack plots of all eyeblinks recorded in three separate experiments from the same animal. *A*: Eyeblinks in an experiment where CRs were tested immediately following an injection of 1.0 µl of muscimol. CRs were gradually abolished within 15 min of post-injection training. *B*: Eyeblinks in an experiment where post-injection testing was delayed by a 15 min diffusion waiting period. Note that CRs were absent starting from the first post-injection trial. *C*: Control experiment. Injecting 1.0 µl of vehicle did not suppress CR performance. For additional information see the legend of Fig. 2.

2.4.4 Effect of DGG injection in the IO on cell activity in the IN

To examine how blocking fast glutamate receptors in the IO affected cell activity in the IN, three additional rabbits were implanted with injection guide tubes in the contralateral IO and with microelectrodes in the IN ipsilateral to the trained eye. The histological reconstruction revealed that all injection sites in these rabbits were located close to the rostral portion of the dorsal accessory olive (Fig. 1) and all recording sites were located in the IN or in the medial part of the dentate nucleus (Fig. 5).

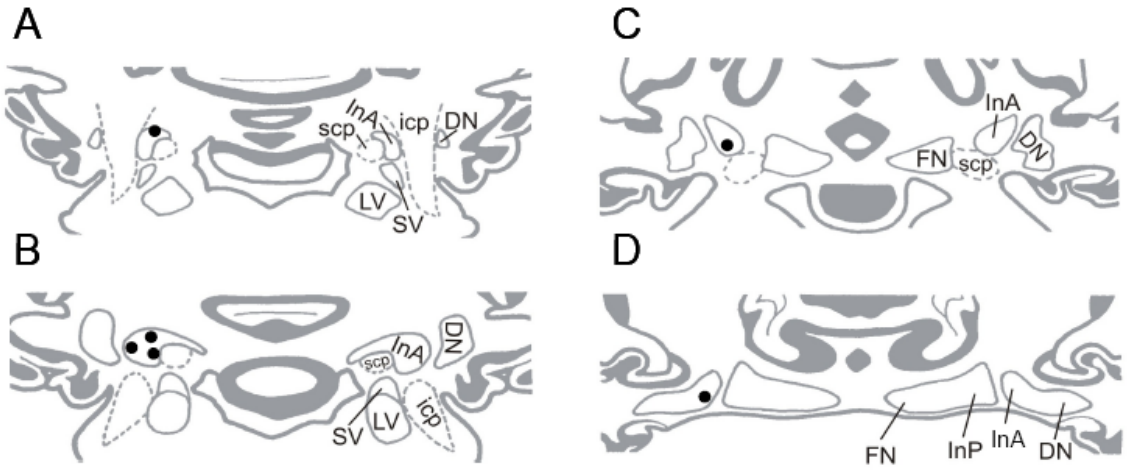
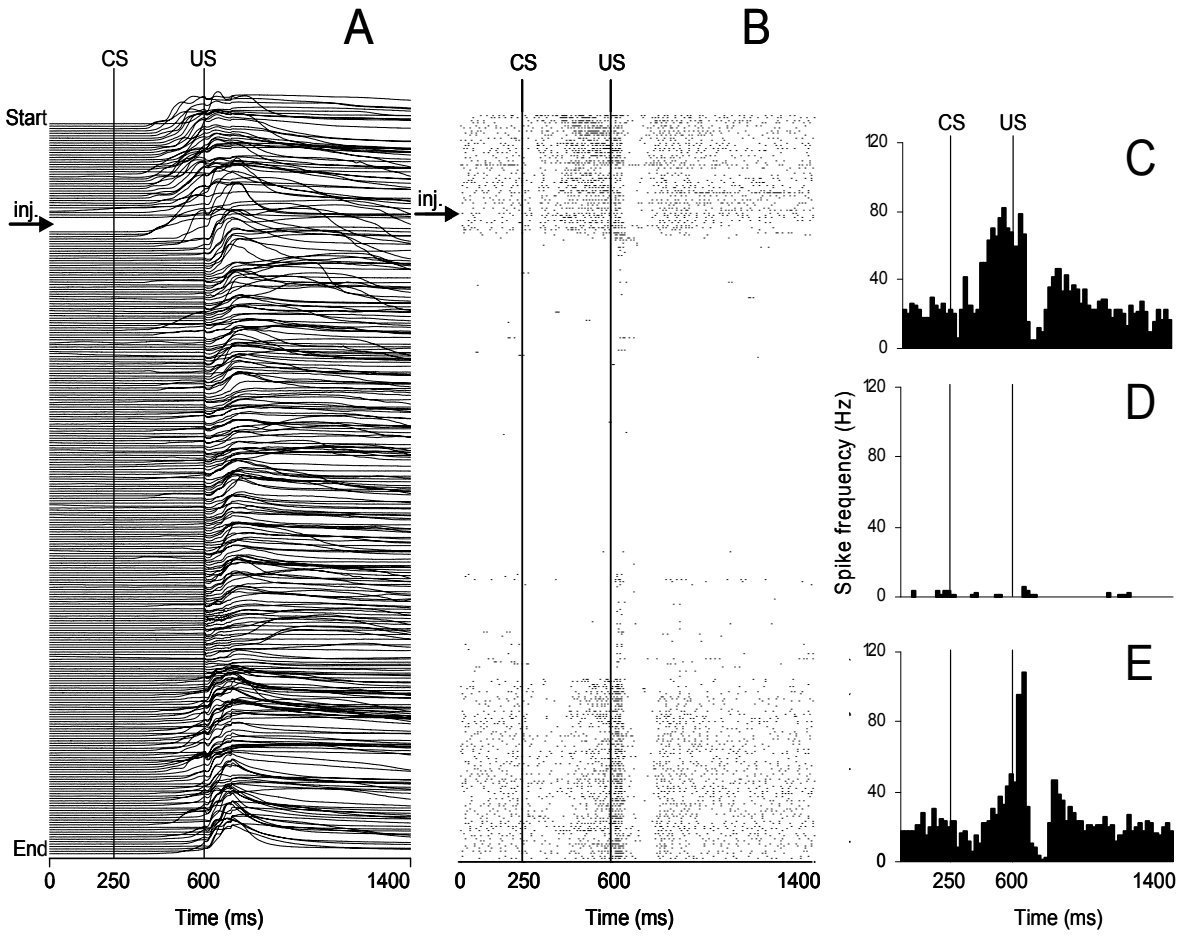


Fig. 5. Reconstruction of cell recording sites in the IN. Out of 9 electrode bundles in three rabbits, five were located in the IN and one in the lateral part of the dentate nucleus. Their position was transferred to a set of standardized coronal sections of the rabbit cerebellar nuclear region. Sections are arranged in rostral-to-caudal order (*A*-*D*). All 38 recorded cells were distributed among the illustrated sites. InA - anterior interposed nucleus; DN - dentate nucleus; LV - lateral vestibular nucleus; SV - superior vestibular nucleus; InP - posterior interposed nucleus; FN - fastigial nucleus; scp - superior cerebellar peduncle; icp - inferior cerebellar peduncle.

Fig. 6. An example of parallel effects of DGG, both on CR performance and on the activity of the task-modulated IN cell. This experiment consisted of 300 trials with the injection administered after 40 trials. *A*: Stack plot of all eyeblinks before and after the DGG injection. CRs were gradually abolished within 3 min following the drug injection. Recovery of CRs was observed at the end of the experiment. *B*: Raster plot of IN cell activity during the same experiment. The experiment starts at the top with each row representing one trial, and dots mark occurrence of action potentials. Shortly after the injection and at the time of behavioral CR abolition, the firing rate and modulation of this cell's activity were severely suppressed. The neuronal activity slowly recovered toward the end of the experiment, with the signs of the recovery apparent before the recovery of behavior in A. *C*: a peri-stimulus histogram of the same IN unit constructed for 40 trials before the injection. This cell expressed a strong excitatory response during the CS-US interval followed by a post-US inhibition. *D*: peri-stimulus histogram of the same cell as in *B* and *C* during 40 post-injection trials when behavioral CRs were abolished and cell activity was suppressed. *E*: peri-stimulus histogram of the same cell during the last 40 trials when partial recovery of behavior and cell activity were observed. Bin width for histograms in *C, D,* and *E* is 20 ms. CS, onset of conditioned stimulus; US, onset of unconditioned stimulus.

Fig. 6.


A
B
C
D
E
CS
US
Start
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inj.
0
250
600
1400
Time (ms)
Spike frequency (Hz)
120
80
40

Figure 6 illustrates parallel effects of injecting 1.0 µl of DGG on CR expression and IN unitary activity. Within this one representative experiment, the IN unit exhibited a combination of excitatory and inhibitory responses to the CS and US. The DGG injection in the IO abolished CR expression (Fig. 6 A) while severely suppressing the baseline firing rate and modulation of the IN neuron (Figs. 6 B-D). Toward the end of the session the behavioral effect partially recovered (Fig. 6 A), which was also accompanied by a partial recovery of the IN neuronal firing rate and modulation (Figs. 6 B, E).

In total, 21 neurons were successfully held and recorded during DGG injections and 17 neurons during injections of vehicle. For the DGG cell population, the mean baseline firing rate decreased from 27.0±1.6 Hz pre-injection to 9.6±2.1 Hz in the first 10 trials post-injection, then to 2.8±0.6 Hz by the third post-injection block (Fig. 7 A); this decrease was significant as shown by comparing the pre-injection rate with the stabilized activity during blocks 7-10 (blocks 3-6 post-injection) ($F_{1,103}$=41.904, $p<0.05$). Significant recovery of the baseline firing rate occurred during the last 40 trials ($F_{1,103}$=5.971, $p<0.05$), but was incomplete relative to the pre-injection level ($F_{1,103}$=16.238, $p<0.05$). Activity of IN neurons was not affected by control injections of vehicle as the mean firing rate remained stable across all 10-trial blocks (Fig. 7 A).

The DGG-induced decrease of IN neuronal activity slightly preceded and coincided with a gradual abolition of behavioral CRs. Mean CR incidence significantly decreased from 93.1±1.6% pre-injection to 14.4±2.2% in blocks 7-10 ($F_{1,37}$=95.719, $p<0.05$, Fig.7 B). Similar to the neuronal activity, partial recovery of CR incidence was observed in the last 40 trials of the recording session (Fig. 7 B), which was significant when compared to blocks 7-10 ($F_{1,103}$=22.398, $p<0.05$), but was incomplete relative to the pre-injection level

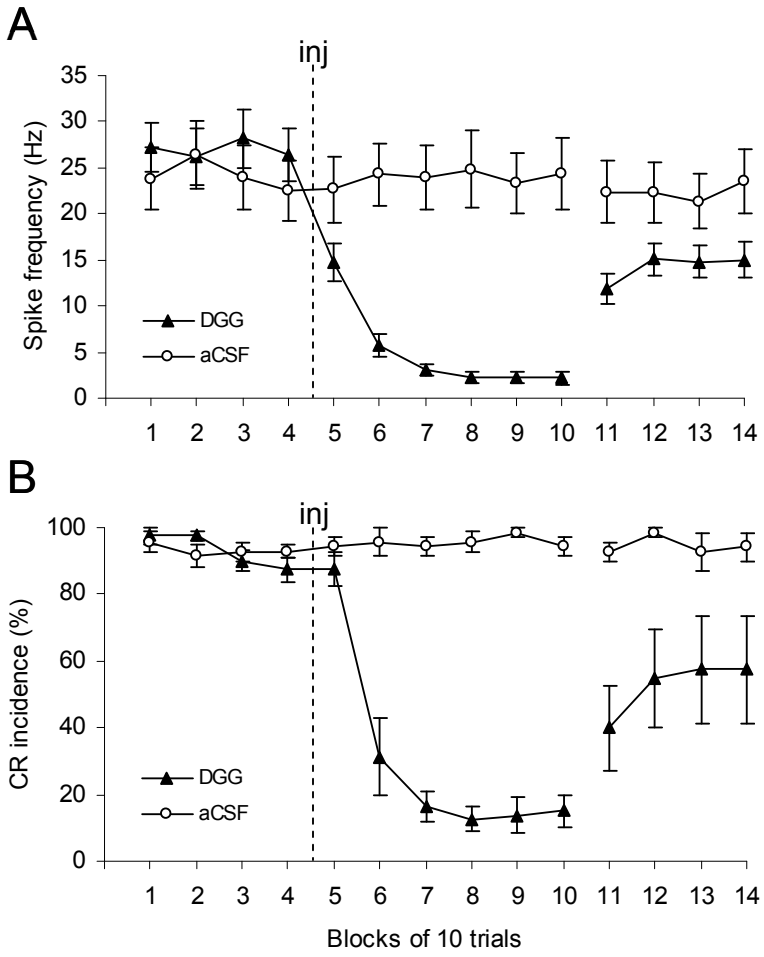

Fig. 7. Group effects of DGG on CR performance and on IN activity during the first six post-injection blocks of trials and the last four blocks of trials. The last four blocks of trials are separated from the rest of the plot by a gap. *A*: Effects of DGG on mean baseline activity (±SE) of IN cells (n=21). Spike frequency of IN cells gradually decreased within the first two blocks after injection of DGG into the IO (▲), and it was almost completely suppressed during the following four post-injection blocks of trials. The cell activity partially recovered during the last four blocks of the experiment. Injection of aCSF into the IO (○) had no effect on IN firing rate (n=17). *B*: Effects of DGG (▲) on mean CR incidence (±SE; n=3) in the same set of experiments as depicted in A. CRs were gradually abolished and then partially recovered by the end of testing. This behavioral effect parallels changes in IN activity shown in A. Control injections of vehicle (○) did not affect CR performance.

($F_{1,103}$=25.513, $p<0.05$). These observations were contrasted with control experiments on the same subjects, in which injections of aCSF did not affect CR incidence (Fig.7 A). The block-by-block analysis of individual cell and CR incidence changes revealed that in most neurons, the DGG-induced suppression of activity preceded the suppression of CRs. Baseline activity of fifteen neurons decreased to less than 50% of their pre-injection level during the first post-injection block, before CR performance declined significantly in those particular experiments. Activity of four cells changed in parallel with the developing CR suppression, and only two cells began to change their activity after changes in CR incidence were detected.

2.5 Discussion and Conclusions

The data presented here demonstrate that activating GABA-A receptors or blocking the glutamate AMPA/kainate and NMDA receptors in the rostral parts of the inferior olive abolish the expression of well-learned eyeblink CRs in the rabbit. Both muscimol and DGG abolished CRs after a variable time delay, ranging from an immediate effect to approximately 15 minutes post-injection. The most important finding of the present study was that the gradual disappearance of CRs was a function of time rather than of post-injection training. This was demonstrated by the absence of CRs when the delivery of stimuli was delayed to allow for drug diffusion. Recordings of IN neuronal activity during the block of glutamate receptors in the IO further suggest that CR abolition was associated with a significant decrease of tonic activity in the deep cerebellar nuclei.

2.5.1 Involvement of the IO in expression of conditioned eyeblinks

A unique feature of eyeblink conditioning in the rabbit is its dependency on the intermediate cerebellum. Studies from several laboratories demonstrated that lesioning or reversible inactivation of the cerebellar interposed nuclei ipsilateral to the trained eye abolishes previously learned responses and also prevents acquisition of new CRs [20, 29, 27, 6, 14]. The present data extend these findings by showings that either blocking the fast glutamate receptors or activating GABA-A receptors in the contralateral IO also abolishes CRs. This finding is consistent with CR suppression reported following IO lesioning [30] or lidocaine inactivation [28]. We also found that the onset of the behavioral effect varied. In some animals, CRs were abolished immediately following the injection. In most cases, however, this effect was delayed for a variable length of time, creating the impression of an extinction-like gradual decrease of CR incidence. To test whether this delay would depend, similar to behavioral extinction, on the post-injection presentation of stimuli, we re-examined the same animals in a waiting period test. In this test, the post-injection training was delayed by a waiting period equal to the previously observed delay of behavioral effects. Following the waiting period, CRs were absent when stimulation was resumed. This finding revealed that CR abolition following muscimol and DGG injections does not require presentation of stimuli and therefore it can not be interpreted as analogous to behavioral extinction. Consequently, the most likely explanation for delayed muscimol and DGG effects was slow drug diffusion to the facial representation within the IO.

The IO is somatotopically organized with respect to its somatosensory inputs. The parts of the IO receiving somatosensory input from the contralateral facial area are the medio-rostral parts of the dorsal accessory olive and the dorsal lamella of the principal olive

[9, 26]. Our conclusion that the timing of CR abolition depends on the rate of drug diffusion to the facial representation in the IO is consistent with the distribution of our injection sites (Fig. 1). Animals having the fastest onsets of behavioral effects were those injected closest to the rostro-medial parts of the dorsal accessory IO (Fig. 1, sections D and E). On the other hand, rabbits that were injected in the most rostral locations (Fig. 1 A, B) displayed the most delayed effects and required higher volumes of muscimol to achieve complete CR block. An important contribution of the present study is its use of synaptically acting drugs to exclude any possible participation of fibers of passage in the observed behavioral effects. Could it be, though, that both muscimol and DGG spread to some other extra-olivary, CR-related neurons? This seems unlikely. The closest relevant structures in this area are the facial and spinal trigeminal nuclei [12]. Involvement of these nuclei does not fit the pattern of measured effects. First, their inactivation should affect predominantly the ipsilateral eye [15,11,8]. Yet, CRs were abolished on the contralateral side. A second argument against a confounding spread to the facial and trigeminal areas is that the shortest distances from injection sites to these targets are from the most rostral for the facial nucleus, and from the most lateral and dorsal for the trigeminal nucleus. As indicated above, the most rostral injections were the least effective in affecting CRs. This, together with preserved unconditioned responses, is inconsistent with significant involvement of the facial nucleus. Also, injection sites closest to the spinal trigeminal nucleus and medullary eyeblink premotoneurons [12] would be locations dorsal to our present injection sites. However, injections placed more dorsal during the functional mapping phase of the experiment had little or no effect on CRs, indicating that if drug spread to the spinal trigeminal nucleus and/or medullary eyeblink premotoneurons, it was not primarily responsible for CR

suppression. Since there is no other potentially confounding target in the area, we conclude that CR abolition was most likely due to suppressing of activity in the rostral parts of the dorsal accessory and principal IO.

2.5.2 The mechanisms for IO inactivation effects

Electrophysiological results of the present study offer an important insight into the possible mechanism of IO inactivation-induced CR abolition. Early studies demonstrated that permanent lesioning or reversible inactivation of the IO has profound effects on tonic activity of the cerebellum. Specifically, removing the IO input to the cerebellum dramatically increased the simple spike frequency of inhibitory Purkinje cells [22], whose increased activity suppressed the activity of deep cerebellar nuclei [3]. Interestingly, reduced activity in the cerebellar nuclei also suppresses the activity in one of their main targets, the red nucleus [5]. These data suggested that blocking the IO could produce a wave of tonic changes altering the functional state of the cerebellum and propagating to cerebellar efferent targets. Our neuronal recording data show for the first time that a similar process occurs in the rabbit eyeblink conditioning preparation. The sequence of related events is illustrated in Fig. 8. We discovered that blocking fast glutamate neurotransmission in the IO dramatically reduces neuronal activity in the cerebellar IN. Inactivating the IO produced behavioral effects and changes in neuronal activity that were strikingly similar to those produced by a direct inactivation of the IN [1]. Since the normal activity of IN neurons is required for CR expression, the suppression of IN activity seems to be causing the IO inactivation-induced CR abolition. Consistent with this notion, the decline of IN activity in the present experiment slightly preceded CR abolition.

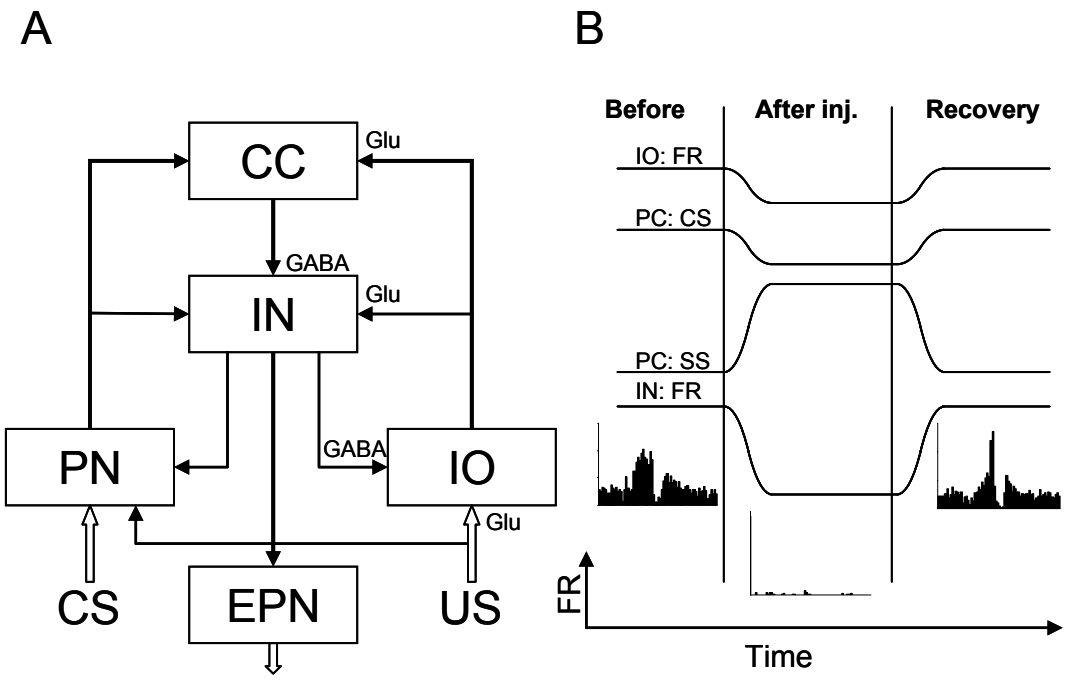

Fig. 8. Schematic of circuits affected by the IO inactivation and of the related cascade of changes of tonic activity. **A** – Simplified schematic of the intermediate cerebellum-related parts of eyeblink conditioning circuits. Boxes represent involved structures and arrows represent axonal connections. Connections labeled Glu are excitatory and mediated by glutamate. Connections labeled GABA are inhibitory and GABA mediated. In the present experiment, inferior olive (IO) was inactivated by blocking fast glutamate receptors or by activating GABA-A receptors. Recordings of the accompanying changes of single-unit activity were performed in interposed nuclei (IN). CS – conditioned stimulus input; US – unconditioned stimulus input; PN – pontine nuclei; CC – cerebellar cortex; EPN – eyeblink pre-motoneurons. **B** – Idealized schematic of the IO inactivation-induced tonic changes that suppressed the cerebellar output and abolished CRs. Injections of DGG in the IO suppressed the firing rate of IO neurons (IOFR). This decreased the incidence of complex spikes in Purkinje cells of the cerebellar cortex (PC:CS). We speculate that this produced a dramatic increase of Purkinje cell's simple spike firing rate (PC:SS). The increased firing of GABA-releasing Purkinje cells then inhibited the activity of IN neurons (INFR), which resulted in the decrease of the drive to eyeblink pre-motoneurons and CR abolition. FR – firing rate.

The present results are pertinent to ongoing interest in the concept that the IO provides the cerebellum with trigeminal US signals that reinforce cerebellar learning. The concept suggests that blocking the trigeminal input to the IO should induce CR extinction [21]. Since blocking AMPA receptors in the IO eliminates the IO response to facial stimulation [17], our DGG injections most likely blocked this IO US input. We found, however, that injecting the IO with DGG rendered the cerebellum dysfunctional, making it impossible to observe effects postulated by cerebellar learning. Could it be that a more selective block of specific receptor types, such as AMPA receptors, could suppress sensory inputs to the IO without modifying tonic cerebellar activity? Although one study seems to support this possibility [21], we were not able to replicate it [24]. Nevertheless, the IO inactivation-induced tonic shift in cerebellar tonic activity does not exclude participation of the IO in learning. It is possible that the IO performs both the tonic-regulatory and error-signaling functions. One of the main conclusions of the present study is that because of associated tonic effects, IO inactivation methods are ill-suited for testing the functional significance of IO sensory signals.

Even though we did not record IN activity during muscimol injections, data from reduced preparations indicate that tonic GABA-ergic inhibition of the IO reduces its activity [18], which is known to strongly elevate Purkinje cell activity [4]. Since increased Purkinje cell activity would be expected to suppress the firing of IN neurons, it is likely that direct muscimol injections in the IO abolish CRs via these same cerebellar mechanisms.

In summary, the present data suggest that the IO has a powerful influence on tonic activity in the intermediate cerebellum and that CR abolition following IO inactivation by DGG or muscimol is a consequence of suppressing neuronal activity in the IN. This

conclusion confirms earlier speculations [28,30], and it offers a feasible mechanism to explain results of prior attempts to block the IO input to the cerebellum. An intriguing question is whether tonic phenomena triggered by IO inactivation take place in normal physiological states. Since the IO receives inhibitory inputs from cerebellar and extra-cerebellar sources [10], it is plausible that tonic inhibition of the IO could be important for setting tonic functional states of the cerebellum, or for gating the cerebellar output flow in normal animals. It should be emphasized that tonic phenomena associated with changes in IO activity do not preclude other functions, such as providing the cerebellum with error or reinforcing signals [13,25]. However, testing these concepts will require new techniques for blocking sensory inputs to the IO without altering cerebellar tonic activity.

2.6 Acknowledgements

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CHAPTER 3. CEREBELLAR DYSFUNCTION EXPLAINS THE EXTINCTION-LIKE ABOLITION OF CONDITIONED EYEBLINKS FOLLOWING NBQX INJECTIONS IN THE INFERIOR OLIVE

A paper is accepted by *The Journal of Neuroscience*

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3.1 Abstract

Classical conditioning of the eyeblink response is a form of motor learning that is controlled by the intermediate cerebellum and related brainstem structures. The inferior olive (IO) is commonly thought to provide the cerebellum with a "teaching" unconditioned stimulus (US) signal required for cerebellar learning. Testing this concept has been difficult because the IO, besides its putative learning function, also controls tonic activity in the cerebellum. Recently it was reported that inactivation of AMPA/kainate receptors in the IO produces extinction of conditioned responses (CRs), suggesting that it blocks the transmission of US signals without perturbing the functional state of the cerebellum. However, the electrophysiological support for this critical finding was lacking, mostly because of methodological difficulties in maintaining stable recordings from the same set of single units throughout long drug injection sessions in awake rabbits. To address this critical issue, we used our microwire-based multiple single-unit recording method. The IO in trained

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rabbits was injected with the AMPA/kainate receptor blocker, NBQX, and its effects on CR expression and neuronal activity in the cerebellar interposed nuclei (IN) were examined. We found that NBQX abolished CR expression and that delayed drug effects were independent of the presentation of the conditioned stimulus (CS) and were therefore not related to extinction. In parallel to these behavioral effects, the spontaneous neuronal activity and CR-related neuronal responses in the IN were suppressed, suggesting cerebellar dysfunction. These findings indicate that testing the IO's role in learning requires methods that do not alter the functional state of the cerebellum.

3.2 Introduction

Classical conditioning of the eyeblink response is a form of motor learning that critically depends on the intermediate cerebellum. Lesioning or inactivation of the interposed nuclei (IN, the output of the intermediate cerebellum) prevents acquisition of new conditioned responses (CRs) as well as the expression of previously acquired CRs (McCormick and Thompson, 1984; Yeo et al., 1985; Welsh and Harvey, 1989; Bracha et al., 1994; Krupa and Thompson, 1997). A popular explanation for the CR's cerebellar dependency is the cerebellar learning hypothesis. According to this concept, the intermediate cerebellar cortex, and to a lesser degree the IN, receive information about the conditioned stimulus (CS) via mossy fibers and about the unconditioned stimulus (US) via climbing fibers. It has been postulated that during learning the interactions between mossy and climbing fiber inputs at points of their convergence induce plastic changes that make the cerebellum more responsive to mossy fiber CS input. The enhanced responses to the CS are thought to generate CRs (Marr, 1969; Thompson, 1986).

The inferior olive (IO), which is the exclusive source of cerebellar climbing fibers, is a pivotal structure within the cerebellar learning hypothesis framework. Based on this proposal, the IO provides the cerebellum with a "teaching" US signal. If so, blocking the IO input to the cerebellum should prevent CR acquisition and it should also extinguish previously learned CRs (McCormick et al., 1985; Mintz et al., 1994). Confirming these predictions experimentally has been difficult because blocking the IO input to the cerebellum produces incapacitating shifts in tonic activity within eyeblink circuits. Inactivating the IO increases the firing rate of cerebellar Purkinje cells, which in turn inhibit cerebellar output nuclei (Montarolo et al., 1982; Benedetti et al., 1983; Batini et al., 1985). This physiological mechanism interferes with testing the learning-related function of the IO. For example, when examining whether IO inactivation extinguishes CRs, we provided evidence that the GABA-A agonist, muscimol, and the broad-spectrum glutamate receptor antagonist, DGG, each abolished CRs not because of extinction, but simply due to tonic dysfunction of cerebellar circuitry (Zbarska et al., 2007). However, in a separate study, Medina et al. (2002) reported the intriguing finding that injecting the AMPA/kainate receptor antagonist, NBQX, in the IO suppressed CRs in an extinction-like fashion. Did injecting the IO with NBQX affect US signals selectively while preserving normal tonic activity of the cerebellum? This is a pivotal question since an affirmative answer would strongly support an IO "teaching" function. To address this issue, we used an advanced method of long-term single-unit recording in pre-trained rabbits while we microinjected NBQX in the IO in order to assess simultaneously the effects of this manipulation on CR expression and the responses and tonic activity of IN neurons. Here we report that blocking AMPA receptors in the IO abolishes CRs by suppressing both the tonic and CR-related neuronal activity in the IN and that the delayed

(extinction-like) behavioral effects of NBQX most likely stem from the diffusion of injected drug.

3.3 Materials and Methods

3.3.1 Subjects

Fifteen male, New Zealand White Rabbits (Harlan, Indianapolis, Indiana), weighing 2.5-3.0 kg (3-4 months old at the beginning of experiments), were used in these studies. Twelve rabbits were included in a study examining the effects of IO inactivation on CR expression (IO injection group). Four animals were used in experiments testing the effects of IO inactivation on CRs and on neuronal activity in the IN (IN recording group). Rabbits were housed individually on a 12/12 hr light/dark cycle and provided with food and water *ad libitum*. All experiments were performed in accordance with the National Institutes of Health's "Principles of Laboratory Animal Care" (publication No. 86-23, revised 1985), the American Physiological Society's "Guiding Principles in the Care and Use of Animals," and the protocol approved by Iowa State University's Committee on Animal Care.

3.3.2 Surgery

Before any training all animals were implanted either with an injection cannula or with both an injection cannula and recording hardware using sterile surgical techniques. Rabbits were anesthetized with a mixture of ketamine (50 mg/kg), xylazine (6 mg/kg), and acepromazine (1.5 mg/kg) and placed in a stereotaxic apparatus with lambda positioned 1.5 mm below bregma. After exposing the skull, three stainless steel anchor screws and a 28-gauge injection guide tube were implanted. For targeting anterior parts of the right IO, the

following stereotaxic coordinates (relative to lambda) were used: AP = x - (0.69x + 4.5) mm anterior to lambda (x is horizontal distance between bregma and lambda in mm), ML = 1.0 mm lateral to lambda, and DV = 22.4 mm ventral to lambda. The patency of the injection guide tube was protected between experiments by a 33-gauge stainless steel stylet.

The four rabbits in the IN recording group were also implanted with a microelectrode array/micromanipulator assembly targeting the left IN as in Aksenov et al. (2004). The manipulator contained three bundles of microwire electrodes totaling 14 electrodes (stainless steel, 18 μm diameter, Formvar insulation). The microwires were connected to a 15-pin connector embedded in dental acrylic. The stereotaxic coordinates for the IN implant were: AP = x - (0.69x + 4.8) mm anterior to lambda (x is distance between bregma and lambda in mm), ML = 5.0 mm lateral to lambda, and DV = 14 mm ventral to lambda. The guide tube, manipulator, connector, anchor screws, and a small Delrin block designed to accommodate an air-puff delivery nozzle and an eyeblink sensor were secured in place with dental acrylic. Following surgery, the manipulator and injection guide tube were protected with removable Delrin caps. All animals were treated with antibiotics during a 5-day post-surgical recovery period.

3.3.3 Training procedures

One week post-surgery, all rabbits were first adapted to a restraint box in a sound-attenuated experiment chamber for 3 days, 30 min per day. Head movements were not restricted either during adaptation or during experiments. Adapted animals were conditioned in the standard delay classical conditioning paradigm until they reached 90% of conditioned responses (CRs) for at least two consecutive days. The conditioned stimulus (CS) was a

binaural 450-ms, 80-dB SPL, 1-kHz tone, superimposed on a 70-dB white noise. As the unconditioned stimulus (US), a 100-ms, 50-psi (at the source) air puff was applied to the left eye. The inter-stimulus interval was 350 ms. The inter-trial interval varied pseudo-randomly from 15 to 25 s. Rabbits had one conditioning session of 100 paired trials per day until they reached 90% of CRs for two consecutive days.

3.3.4 Injection procedures

Before each injection experiment a 33-gauge stainless steel needle was inserted in the intracranial guide tube. The injection needle was connected to a 10-μl Hamilton syringe using transparent Tygon tubing. The pressure microinjections were performed manually at a rate of 0.5 μl/min. The injected volume was monitored by observing the movement of a small air bubble relative to gradation marks on the transparent tubing. During injection sessions, 40 trials of paired stimuli were applied prior to the injection to assure that needle insertion had no effect on CR performance. Injections were not administered if rabbits had less than 85% CRs during the pre-injection baseline period. NBQX (Sigma-Aldrich) was dissolved in artificial cerebrospinal fluid (aCSF, Medina et al., 2002) and its pH was adjusted to 7.4±0.1.

Functional mapping and non-waiting experiment. To increase the yield of experiments, we first determined the effective vertical placement of NBQX injections by gradually increasing the injection depth. During the first mapping session, NBQX (0.5 μl, 150 μM) was injected at the depth of the guide tube tip. Mapping sessions consisted of 40 pre-injection trials and 160 post-injection trials, which were administered immediately following the initial NBQX injection. Similar to Medina et al. (2002), additional 0.5 μl

injections were administered at 5 min intervals until an effect of the drug on CRs was observed or until a total of eight injections was reached; the total volume of NBQX ranged from 0.5 to 4 μl among rabbits. If no effect on CR incidence was observed, the depth of injection was adjusted, progressing 0.5 mm deeper the next day. This daily increase of injection depth was repeated until NBQX completely abolished CRs, or until the needle reached the base of the skull. The last functional mapping session was included in the data analysis as the *non-waiting experiment*.

The waiting experiment. The next day following the non-waiting test, all rabbits were tested in a waiting experiment, consisting of three parts. In the first part, 40 trials were delivered to assess normal pre-injection CR performance. The second part was a waiting period designed to allow for effective drug diffusion before CS-US stimulation was resumed. During this period the same amount of NBQX was administered using the same injection schedule as in the previous non-waiting experiment. No stimuli were presented during the waiting period and its duration was set to last 5 min longer than the time it took to abolish CRs in the non-waiting experiment in each particular animal. Following the waiting period, CR performance was tested in 160 trials.

Controls. During control experiments, a corresponding volume of vehicle (aCSF) was administered into the IO using the same injection and paired stimulation schedule as in NBQX experiments.

Injections in the IN recording group. The four rabbits in the IN recording group were repeatedly tested both in waiting and non-waiting experiments using NBQX and aCSF injections. To capture the behavioral and neuronal recovery, we extended the post-injection observation period to a maximum of 260 trials. If the behavioral CR recovery occurred and

was maintained for 40 trials (waiting experiment) or 30 trials (non-waiting experiment), the recording session was terminated before 260 post-injection trials were completed. Once the effect of NBQX had been tested on a group of isolated single units, the microwire electrodes were advanced deeper to find an additional group of IN neurons. If new neurons were isolated, then another set of NBQX and aCSF experiments were repeated on these new units. This procedure was repeated until no more new units could be isolated, or until the NBQX injections became ineffective due to gliotic scarring around the IO injection site. Not all IN units were paired with control injections of aCSF because of the need to limit the number of needle insertions at the injection site, and because some units degraded or were lost before they were ready to be tested against aCSF.

3.3.5 Data recording and analysis

In the IO injection group, the eyeblink was recorded using a new, wide field-of-view infrared sensor (Ryan et al., 2006), which was attached to the rabbit's head implant before each experiment. This arrangement secured the constant position of the sensor relative to the eye, yielding reliable eyeblink recordings without the need for head restraint. The sensor's signal was amplified, digitized at 1 kHz with 12-bit resolution, and displayed and stored on a custom data acquisition system. Data were acquired for 1400 ms in each trial, starting 250 ms before the CS onset. Eyeblink responses beginning between 80 ms after CS onset and up to the US onset were considered CRs when they exceeded the mean of the signal in the 250-ms baseline period by more than five standard deviations (approximately 0.1 mm). This relatively low threshold was selected to capture small responses that could be present during an incomplete drug effect or when the drug effect starts to recover. Spontaneous responses

were defined as trials in which the difference between the maximum and minimum values in the baseline period exceeded 0.5 mm. Any blink exceeding the CR threshold within 80 ms after CS onset was recognized as an alpha response. Trials containing spontaneous blinks or rare alpha responses were stored but were not included in further data analyses. Measurements of eyeblink responses were used for calculations of CR incidence per block of 10 trials in each session.

In addition to the visualization of eyeblink traces on the computer monitor, two video cameras were installed in the experiment chamber to monitor general animal behavior during experiments. One camera provided a front view of the rabbit's head and the other one, configured for infrared viewing, was positioned on the side of the trained eye. This setup proved to be invaluable for monitoring rabbit behavior continuously.

In the IN recording group, multiple single-unit signals from microwire electrodes were fed through a custom miniature 14-channel FET-based preamplifier to a multi-channel differential amplifier system (Grass-Telefactor model 12 Neurodata System). The amplified and band pass-filtered (300 Hz-3 kHz) signal was digitized (25 kHz/channel) using a custom data acquisition system, and was displayed and stored in 1400-ms epochs corresponding to individual trials. Unit discrimination was performed offline using threshold detection followed by a cluster analysis of scatter plots of time and amplitude distances between the peak and valley of individual action potential waveforms. The discriminated data were processed using custom software in addition to a commercial data analysis package (NeuroExplorer, Nex Technologies). Cross-correlation analysis was performed for each unit to eliminate multiple inclusions of the same unit recorded on different wires within a bundle. Raster and peri-event histograms were constructed for each unit. For cells recorded during

the waiting period test, separate histograms were constructed for 30 pre-injection trials, 30 trials during the waiting period while injecting, 30 trials for the post-injection period when stimulation was resumed, and 30 trials at the end of session representing recovery. The criterion for identifying the recovery period was the complete behavioral reappearance of CRs by the end of the experiment or the last 30/40 trials of a 260-trial experiment. In the latter case the behavioral recovery could be incomplete. In each histogram, the baseline firing rate (250 ms before CS onset) and the timing of significant excitatory and inhibitory changes were computed. Cell responses were considered significant if modulation of the firing rate from the CS onset until the end of the trial exceeded the mean baseline ± tolerance limit for two consecutive 20-ms bins. Tolerance limits were computed to capture 95% of the baseline distribution with a probability of 0.95.

Baseline means of individual cell histograms were pooled together within NBQX, NBQX waiting and control experiments and then statistically analyzed. Only units that were isolated reliably throughout an experiment, as judged by the invariance of the shape and size of action potentials, were included in the data analysis.

For the statistical analysis, we used repeated measures ANOVA with the following factors: drug (3 levels: drug, drug with delay, and aCSF) and time/dose (4 levels: before injection, injection-no stimulation, after injection- stimulation resumed, and recovery). The repeated factor was the four 10-trial blocks for non-waiting experiments and three 10-trial blocks for waiting experiments comprising each phase of an experiment. For the analysis of IN group cell activity, the ANOVA included an additional blocking factor (rabbit) to account for multiple cells recorded from the same animal. We generated planned contrasts from the ANOVAs to address *a priori* hypotheses regarding patterns of CR incidence and baseline

activity of IN neurons arising from the interaction between treatments and phases of an experiment. Specifically, using groups of 10-trial blocks, we performed 1-df contrasts between pre-injection, waiting, post-waiting and recovery within and between experiments with no time delay, with time delay, and their controls. All group data are reported as mean ± standard error of the mean. The level of statistical significance was set at $p<0.05$. All statistical analyses were performed using Statsoft Statistica software.

3.3.6 Histology

After all experiments were completed, rabbits were deeply anesthetized with a mixture of ketamine (100 mg/kg), xylazine (12 mg/kg), and acepromazine (3 mg/kg). The injection site was marked by injecting 1 μl of tissue-marking dye. Anesthetized animals were transcardially perfused with 1 L of phosphate-buffered saline followed by 1 L of fixative (10% buffered formalin) and 1 L 10% potassium ferrocyanide in 10% formalin. The potassium ferrocyanide was applied only to IN-recording rabbits, where the location of electrodes was marked by passing 10 μA anodal DC current through each wire for 20 s. Brains were removed and placed in a solution of 30% sucrose and 10% formalin. After one week, brains were sectioned coronally at 50 μm on a freezing microtome. Sections were mounted onto gelatin-coated slides, dried, and stained with either Luxol blue and neutral red (IO injection group) or with ferrocyanide hydrochloride and neutral red (IN recording group). Locations of injection sites were determined using bright field microscopy and transferred to a standardized set of coronal sections of the rabbit medulla. Locations of electrode marks were transferred to a standardized set of coronal sections of the rabbit cerebellar nuclear region.

3.4 Results

3.4.1 Location of injection sites and general observations

A total of 15 rabbits were implanted with an injection cannula in the IO. Three animals were not included in the data analysis because low-concentrations of NBQX (150μM) injected in the IO did not affect CR expression. Of the remaining 12 rabbits, four also had recording electrodes implanted in the IN. The histological reconstruction of IO injection sites revealed that they were located in the rostral portion of the inferior olivary complex, close to the rostral part of the dorsal accessory inferior olive (Fig.1).

All rabbits reached the 2-day 90% CR criterion in 5 to 12 sessions. The mean CR incidence was 91.4±0.5% in the final training session. Once effective injection sites were established through functional mapping, microinjections of NBQX reliably abolished CRs, albeit with a variable delay (see sections 3.2 and 3.3 for additional information). Unlike DGG and muscimol, which in our previous study increased tonic eyelid aperture and affected eye movement and body posture (Zbarska et al., 2007), NBQX injected to the IO did not have any visible side effects.

3.4.1 Effect of NBQX on CR expression

Immediate post-injection test in the non-waiting experiment. Injecting NBQX at effective injection sites abolished CR expression. Typically, rabbits exhibited high CR incidence during pre-injection trials, followed by gradual CR suppression in the post-NBQX infusion period (Fig. 2A). During the individual experiment shown in Fig. 2A, 3.0 μl of

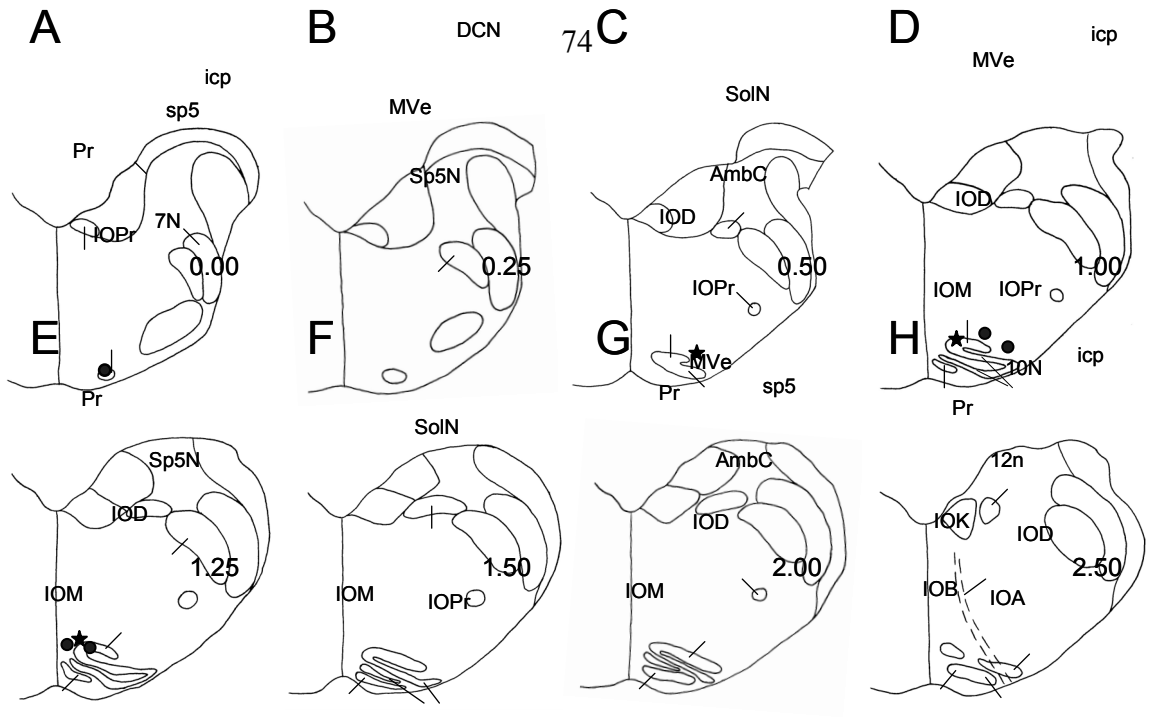


Fig. 1. Location of injection sites in the IO injection group (●, n=8) and in the IN recording group (★, n=4). The identified sites in individual animals were transferred to a set of standardized coronal sections of the rabbit medulla, arranged in rostral-to-caudal order (*A-H*). The anterior-posterior distance in mm from the rostral pole of the IO is on the lower right side of each section. All injection sites were found within or close to the rostral part of the inferior olivary complex. Injections of NBQX at all marked sites abolished CR expression. DAO - dorsal accessory inferior olive; IOM - medial inferior olive; IOPr - principal inferior olive; IOA - subnucleus A of the medial inferior olive; IOB - subnucleus B of the medial inferior olive; IOK - cap of Kooy of the medial inferior olive; Sp5N - spinal trigeminal nucleus; sp5 - spinal trigeminal tract; Pr - prepositus hypoglossal nucleus; 7N - facial nucleus; icp - inferior cerebellar peduncle; MVe - medial vestibular nucleus; SolN - solitary nucleus; Amb - ambiguous nucleus; 12n - hypoglossal nerve; 10N - dorsal motor nucleus of the vagus; DCN - dorsal cochlear nucleus.

NBQX were delivered in six 0.5-µl increments spaced 5 minutes apart. CRs were gradually abolished over the course of about 23 minutes since first injecting NBQX and they did not recover during the rest of the experiment. Injections of the same volume of vehicle at the same site had no effect on CR incidence (Fig. 2C).

The onset timing of CR abolition among rabbits varied from immediate to approximately 35 min post-injection. Group data (n=12) revealed that CR incidence gradually decreased from 90.0±4.3% in the last pre-injection block of 10 trials to 5.0±2.3% in the seventh post-injection block (Fig.3). Complete or partial recovery of CRs by the end of the experiment was observed in six out of twelve rabbits; during the last 10-trial block, mean CR incidence was 31.0±10.1% (Fig.3). This decline of CR incidence contrasted with stable CR performance in control experiments before (93±2.7%) and after (90.3±3.9%) vehicle injections. The repeated measures ANOVA yielded significant drug effects. Planned comparisons revealed a significant effect of NBQX ($F_{1,30}$=254.2, $p<0.001$) when contrasting overall pre- and post-injection CR performances, whereas effects of vehicle were insignificant ($F_{1,30}$=0.3, $p=0.570$).

The effect of lower concentrations of NBQX was tested on one rabbit, which in the non-waiting experiment showed almost immediate abolition of CRs after the first of two 0.5µl injections of NBQX (150µM, Fig. 4A). In this animal, even an NBQX concentration eight-times lower (19µM) quickly abolished CRs with an effect lasting about 50 trials before CRs recovered (Fig. 4B). Also, CR abolition does not always require repeated injections of NBQX. We observed CR abolition after a single NBQX injection in one rabbit and after two injections in two rabbits.

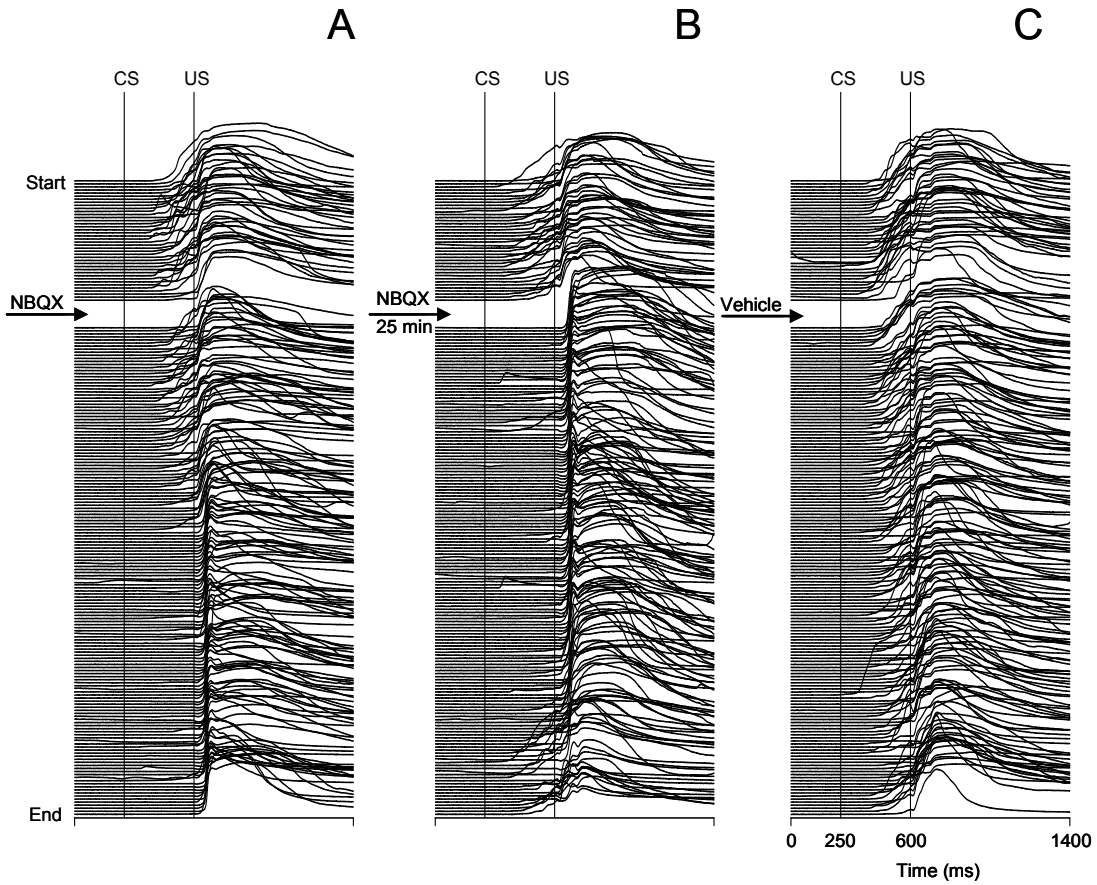


Fig. 2. Individual examples from the same subject showing behavioral effects of microinjecting NBQX and vehicle (aCSF) into the IO. Each stack plot represents a complete printout of eyeblinks from a 200-trial injection experiment. The experiments start at the top, and each eyeblink trace represents one trial. The timing of injections is denoted by an arrow in each stack plot. Conditioned eyeblinks are upward deflections of the signal between the vertical CS and US onset markers. *A*: Eyeblinks in an experiment where CR testing was resumed immediately following the injection of NBQX. Note that CRs were gradually abolished 23 minutes after NBQX. *B*: Stack plot of eyeblink mechanograms from an experiment in which the rabbit was injected with the same amount of drug, but the CR testing was delayed by inserting a waiting period for NBQX diffusion. In this test, CRs were absent immediately after stimuli resumed, indicating that the delayed drug effect in part A was simply a function of time, most likely related to drug diffusion. Note the partial recovery of CRs as the waiting experiment continued an extra 25 min. *C*: Eyeblinks in the control experiment in which injections of the same amount of vehicle (aCSF) did not affect CRs.

The waiting period test. The waiting period test was administered to examine whether the gradual NBQX-induced abolition in the previous test was related to CR extinction or to some other time-dependent process, such as drug diffusion. If the gradual

CR abolition represented CR extinction, then we expected that postponing the CS-US presentation following the NBQX injection should have no effect on the time course of CR suppression. This prediction was not confirmed. Figs. 2B and 4C illustrate that CRs were absent immediately from the beginning of the post-injection test. Clearly, the NBQX-induced CR suppression did not require the presentation of the CS, which is necessary for CR extinction. In most waiting experiments, CRs partially recovered by the end of post-injection testing (Fig. 2B, 4C).

During the waiting period test, mean CR incidence precipitously declined from 96.5±2.6% in the last block of pre-injection trials to 5.0±1.5% during the first 10 post-waiting trials (Fig. 3). The post-waiting decline in CR incidence was significant both when compared to the pre-injection period ($F_{1,30}$=307.7, $p<0.001$) and to the first block of 40 post-injection trials in the non-waiting test ($F_{1,30}$=36.8, $p<0.001$). Because of the extended period of observation during waiting period tests, CRs tended to recover in these experiments, starting from the fifth post-waiting block of trials. This recovery was observed in eight out of twelve rabbits with one of them illustrated in Fig. 2B. In the last 10-trial block after the waiting period test, mean CR incidence in the IO injection group was 55.8±12.2%. During the last 40 trials after the waiting period test, CR incidence had partially recovered to 49.2±11.8%, which was significantly different from the corresponding block of trials in the non-waiting test (20.3±9.0 %, $F_{1,30}$=6.0, $p=0.02$).

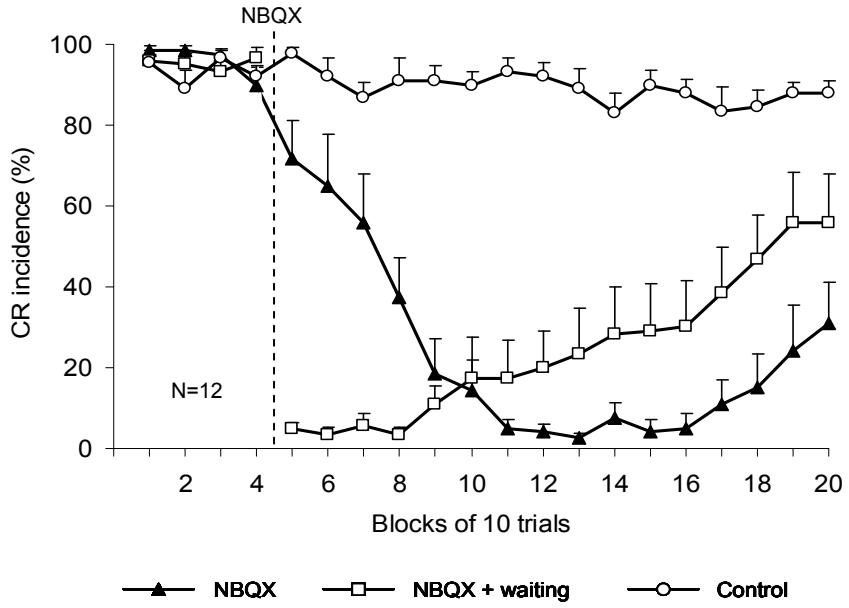


Fig. 3. Effects of NBQX on CR incidence (±SE) in IO injection rabbits (n=12) when tested immediately following the injection (▲) and following the diffusion waiting period (□) are compared to those observed following the control injection of vehicle (○). The vertical dashed line denotes the onset of NBQX injections. Note that up to 6 blocks of trials were required to obtain complete CR abolition in the non-waiting test, while CRs were immediately absent in the first post-injection block of the waiting test. Injection of vehicle (aCSF) did not affect CR incidence.

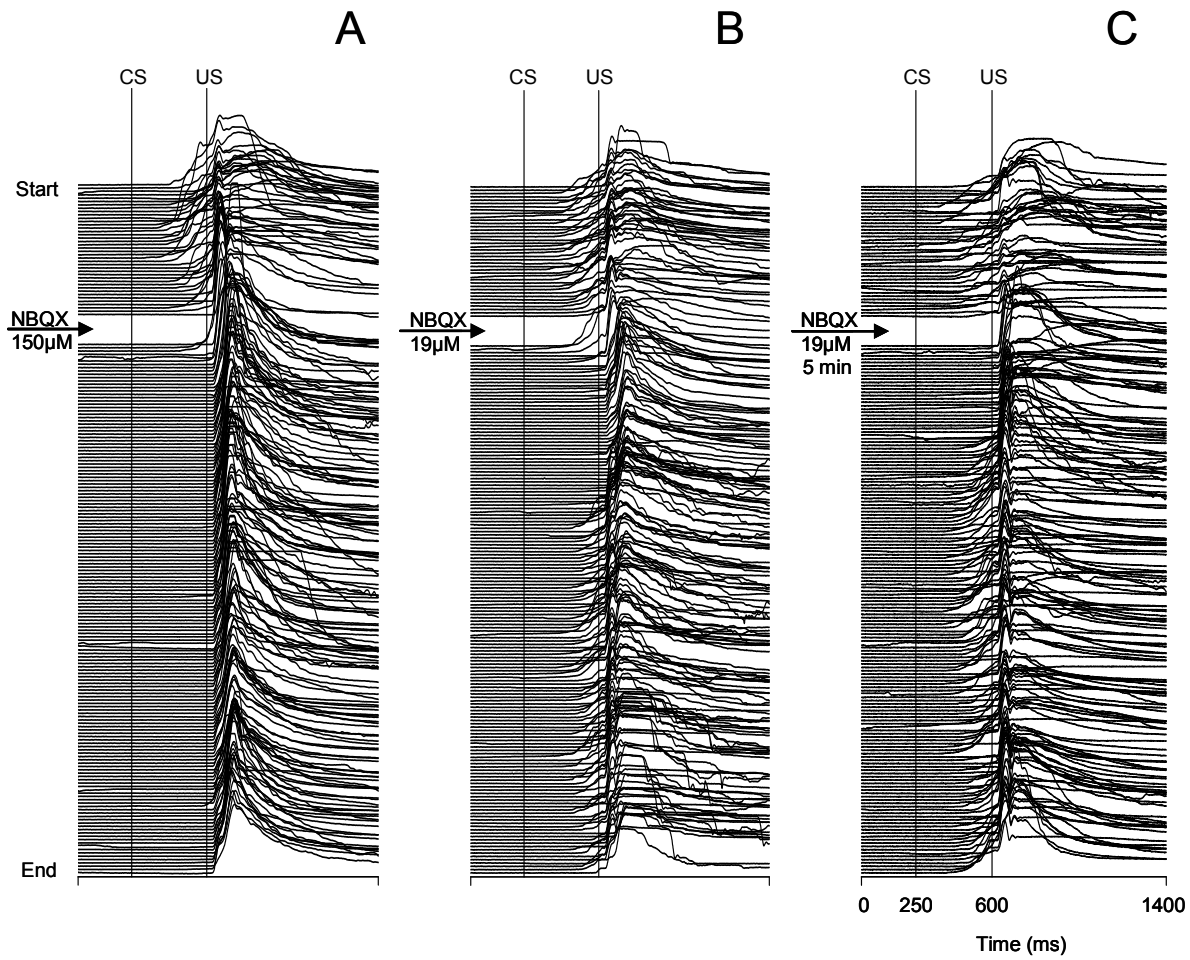


Fig. 4. Stack plots of all eyeblinks recorded in three separate experiments from the same animal showing the behavioral effects of microinjecting different concentrations of NBQX in the IO. *A*: An experiment where CRs were tested immediately following an injection of 1.0 µl of NBQX (150 µM). CRs were almost immediately abolished after 1 min of post-injection training. *B*: Eyeblinks in an experiment where 1.0 µl of 8-times less concentrated NBQX (19 µM) was injected and then an immediate test was performed. CRs were abolished following several post-injection trials, but the duration of the effect was shorter than that obtained with the higher concentration of NBQX shown in A. *C*: Eyeblinks in an experiment where post-injection testing of 19 µM NBQX was delayed by a 5 min diffusion waiting period. Note that CRs were absent starting from the first post-injection trial, and then 10 minutes later they started to recover. For additional plot information, see Fig. 2.

3.4.2 Effect of NBQX injection in the IO on neuronal activity in the IN

Immediate post-injection test. The histological reconstruction performed in the four IN recording rabbits revealed injection sites located close to the rostral portion of the dorsal accessory olive (Fig. 1). All recording sites were located in the IN, with the majority of electrodes in the anterior IN (Fig. 5). In total, 27 neurons were recorded during NBQX injections and 20 neurons during injections of vehicle, all in the non-waiting experiment. Single-unit recordings revealed that the gradual CR abolition induced by NBQX coincided with the gradual suppression of spontaneous firing rates of IN cells (Figs. 6 A, B).

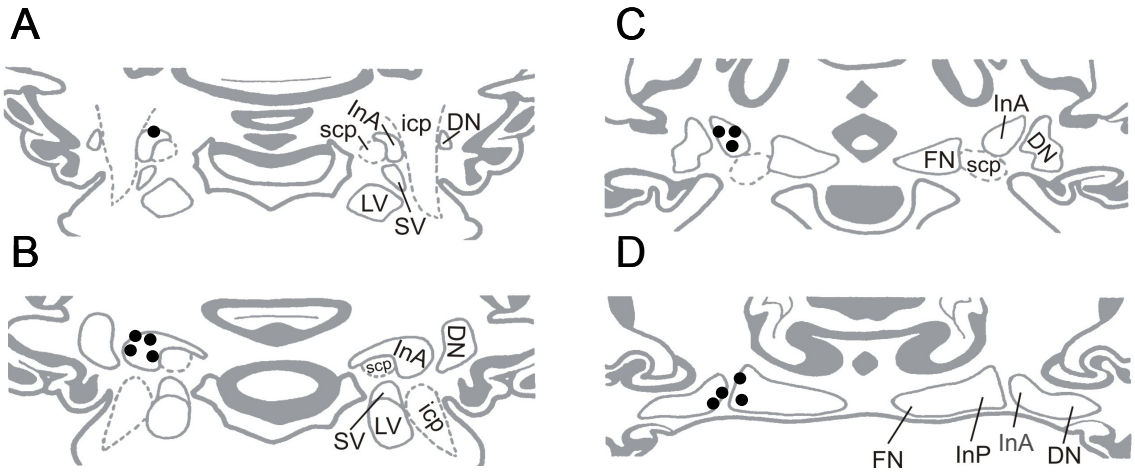


Fig. 5. Reconstruction of cell recording sites in the IN. Out of 12 electrode bundles in four rabbits, eight were located in the anterior IN and four at the caudal border of the InA between the DN and InP. Their position was transferred to a set of standardized coronal sections of the rabbit cerebellar nuclear region. Sections are arranged in rostral-to-caudal order (*A-D*). InA - anterior interposed nucleus; DN - dentate nucleus; LV - lateral vestibular nucleus; SV - superior vestibular nucleus; InP - posterior interposed nucleus; FN - fastigial nucleus; scp - superior cerebellar peduncle; icp - inferior cerebellar peduncle.

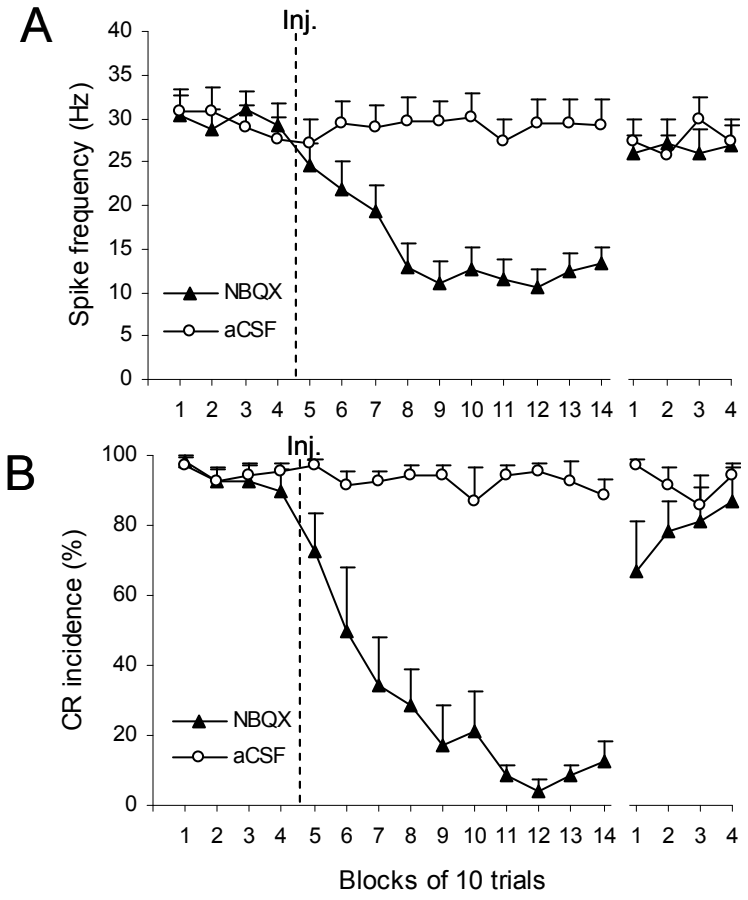


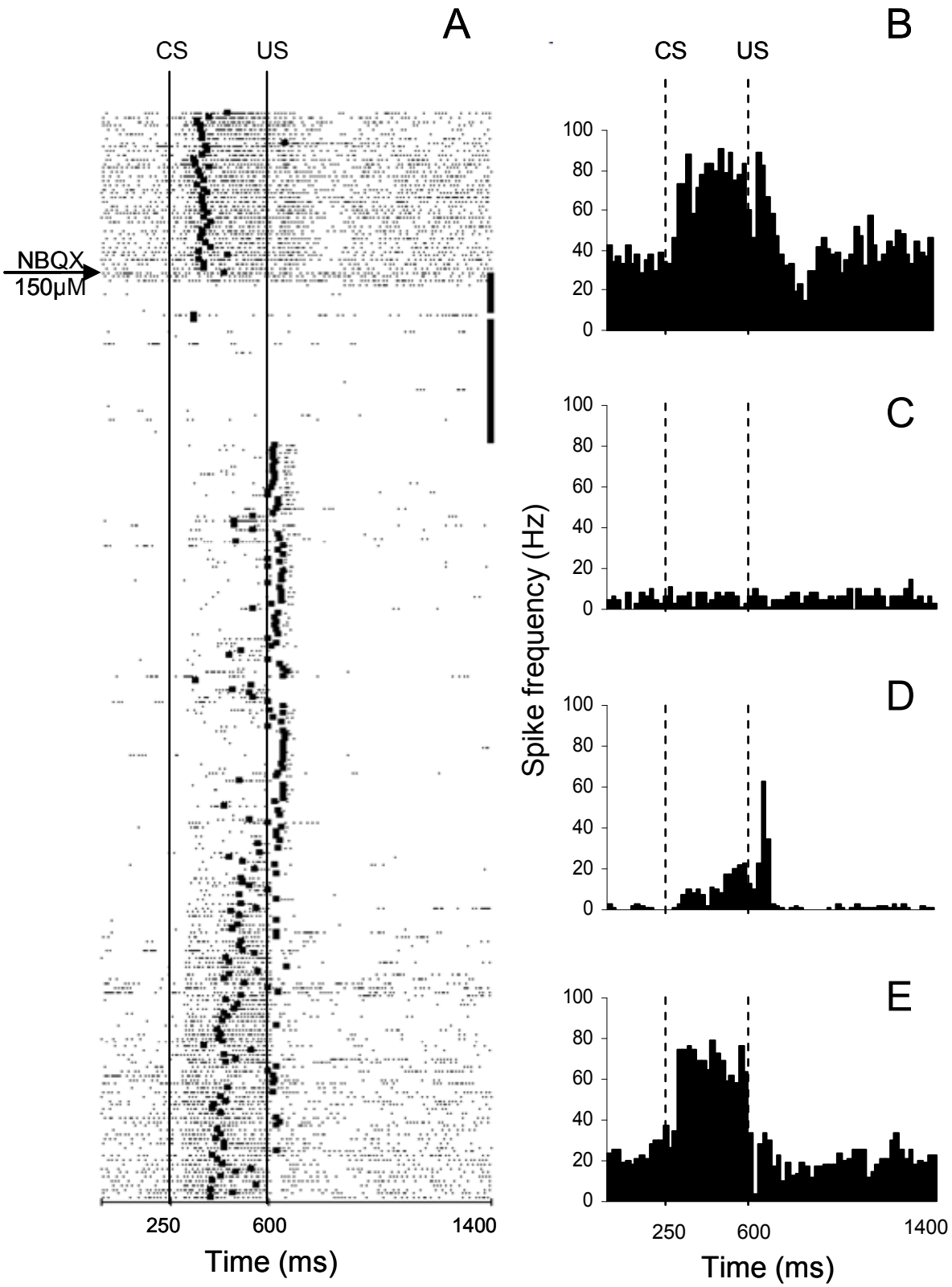
Fig. 6. Group effects of NBQX on CR performance and on IN activity during the non-waiting test. The last four blocks of trials represent the recovery period and are separated from the rest of the plot by a gap. *A*: Effects of NBQX on mean baseline activity (±SE) of IN cells (n=27). The firing rate of IN cells gradually decreased within the first four blocks of trials after the injection of NBQX (▲), and it remained at slightly above 10Hz for the next 60 trials. The spontaneous activity almost completely recovered during the last four blocks of the experiment. Injections of aCSF (○) had no effect on IN firing rate (n=20). *B*: Effects of NBQX (▲) on mean CR incidence (±SE; n=7) in the same set of experiments as depicted in A. CRs were gradually abolished and then partially recovered by the end of testing. This behavioral effect parallels changes in IN activity shown in A. Control injections of vehicle did not affect CR performance.

In this NBQX cell population, the mean baseline firing rate decreased significantly, first from 29.8±2.3 Hz pre-injection to 19.2±3.0 Hz by the third post-injection block of 10 trials, and then to 11.0±2.5 Hz by the fifth post-injection block (Fig. 6 A, Blocks 1-4 vs. 5-14, $F_{1,42}$=70.2 , p<0.001). The baseline firing rate markedly recovered during the last 40 trials (Blocks 5-14 vs. 15-18, $F_{1,42}$= 51.8, p<0.001), and it almost reached its pre-injection level of activity (Blocks 1-4 vs. 15-18, $F_{1,42}$=6.0 , p=0.018). In contrast, the mean firing rate during control experiments remained stable after vehicle-only injections (Fig. 6A, Blocks 1-4 vs. 5-14, $F_{1,42}$=0.030, p=0.864). The time gap between the last 10-trial block of post-injection trials (Fig. 6, Block 14) and the first block of the recovery period varied from 7-27 minutes (20-80 trials).

The NBQX-induced decrease of IN neuronal activity coincided with a gradual decline in mean CR incidence from 93.6±3.9% pre-injection to 34.3±13.8% by the third post-injection block, and then to 4.29±3.0% by the eleventh block (Fig.6B, Blocks 1-4 vs. 5-14, $F_{1,8}$=495.3, p<0.001). Likewise, a behavioral recovery, which paralleled the recovery of neuronal activity during the last 40 trials, was significant when compared to blocks 11-14 ($F_{1,8}$=245.6, p<0.001), but it was incomplete relative to the pre-injection level ($F_{1,8}$=40.0, p<0.001). Control injections of aCSF in the IO had no effect on CR incidence which was 95.0±2.6% and 92.3±3.8% before and after vehicle injections correspondingly (Fig.6B).

The waiting period test. Inserting a waiting period between the NBQX injection and behavioral testing provided a unique opportunity to measure IN activity at this time. As illustrated in an individual example, IN neurons exhibited a strong excitatory response to the CS prior to the onset of the US and a combination of excitatory and inhibitory responses to the US (Fig. 7A, B). The NBQX injection in the IO of this rabbit almost immediately

Fig. 7**.** An example from a waiting period test of the parallel effects of NBQX on CR performance and on the activity of a task-modulated IN cell. This experiment consisted of 260 trials with the two 0.5 µl NBQX injections administered during 40 trials no-stimulation period that began immediately after 40 pre-injection trials. *A*: Raster plot of IN cell activity during the same experiment. The experiment starts at the top with each row representing one trial, and each dot marking the occurrence of an action potential. The black square on each row corresponds to the onset of the eyeblink in that particular trial. The 40 black squares at the end of rows correspond to the no-stimulation waiting period. This cell responded with excitation during the CS-US interval followed by a combined excitatory/inhibitory response to the US. Shortly after the first NBQX injection, the firing rate of this cell's activity precipitously declined. When stimulation was resumed, CRs were abolished immediately, baseline activity remained suppressed, and modulation during the CS-US interval was severely reduced while the relative excitatory modulation to the US became more distinct. The neuronal activity gradually recovered toward the end of the experiment in parallel with the recovery of behavioral CRs. *B-E*: Peri-stimulus histograms of the same IN unit constructed for 40 trials before the injection (*B*), for 40 post-injection waiting trials when stimulation was paused (*C*), for 40 post-waiting period trials when stimulation was resumed (*D*), and for the last 40 trials of the experiment (E). Bin width for histograms in *B-E* is 20 ms. CS, onset of conditioned stimulus; US, onset of unconditioned stimulus.


A
CS
US
NBQX
150μM
250
600
1400
Time (ms)
B
C
D
E
Spike frequency (Hz)
100
80
60
40
20
0
250
600
1400
Time (ms)

severely suppressed the firing rate of the IN unit during the no-stimulation period (Figs. 7A, C). When stimulation was resumed, behavioral CRs were absent. At the same time, the firing rate of this cell remained inhibited and its modulation was altered. The excitatory response to the CS dramatically decreased and the excitatory response to the US became more prominent, albeit shorter-lasting (Figs. 7A, D). Toward the end of the session the behavioral effect gradually recovered (Fig. 7A), accompanied by a partial recovery of the neuronal firing rate and modulation (Fig. 7 A, E).

In total, 88 neurons were successfully held and recorded during the NBQX waiting period test and 32 neurons in vehicle injection controls. Among the 88 NBQX neurons, 79 cells exhibited a significant decrease in baseline activity during the waiting period when compared to the pre-injection level. Of the nine remaining neurons in this group, eight did not change their activity and one neuron significantly increased its firing rate. The mean baseline firing rate of NBQX neurons (n=88) decreased by more than half from 28.1±1.5 Hz pre-injection to 13.1±1.3 Hz by the end of waiting period (Fig. 8 A, Blocks 1-3 vs. 4-6, $F_{1,118}$=77.0, $p<0.001$). When stimulation was resumed, the mean baseline firing rate decreased further to 11.4±1.3 Hz (Blocks 1-3 vs. 7-9, $F_{1,118}$=104.4, $p<0.001$). Recovery of the baseline firing rate occurred during the last 30 trials (Blocks 10-12 vs. 4-9, $F_{1,118}$=66.0, $p<0.001$), but it was incomplete relative to the pre-injection activity (Blocks 1-3 vs. 10-12, $F_{1,118}$=11.3, $p=0.001$). The time gap between the last 10-trial block of post-injection trials (Fig. 8, Block 9) and the first block of the recovery period varied from 10-43 minutes (30-130 trials). The firing rate of IN neurons was not affected by control injections of vehicle, as the mean firing rate remained stable before (30.0±2.4%) and after (27.3±2.3%) injections (Fig. 8 A, Blocks 1-3 vs. 7-9, $F_{1,118}$=0.736, $p=0.393$).

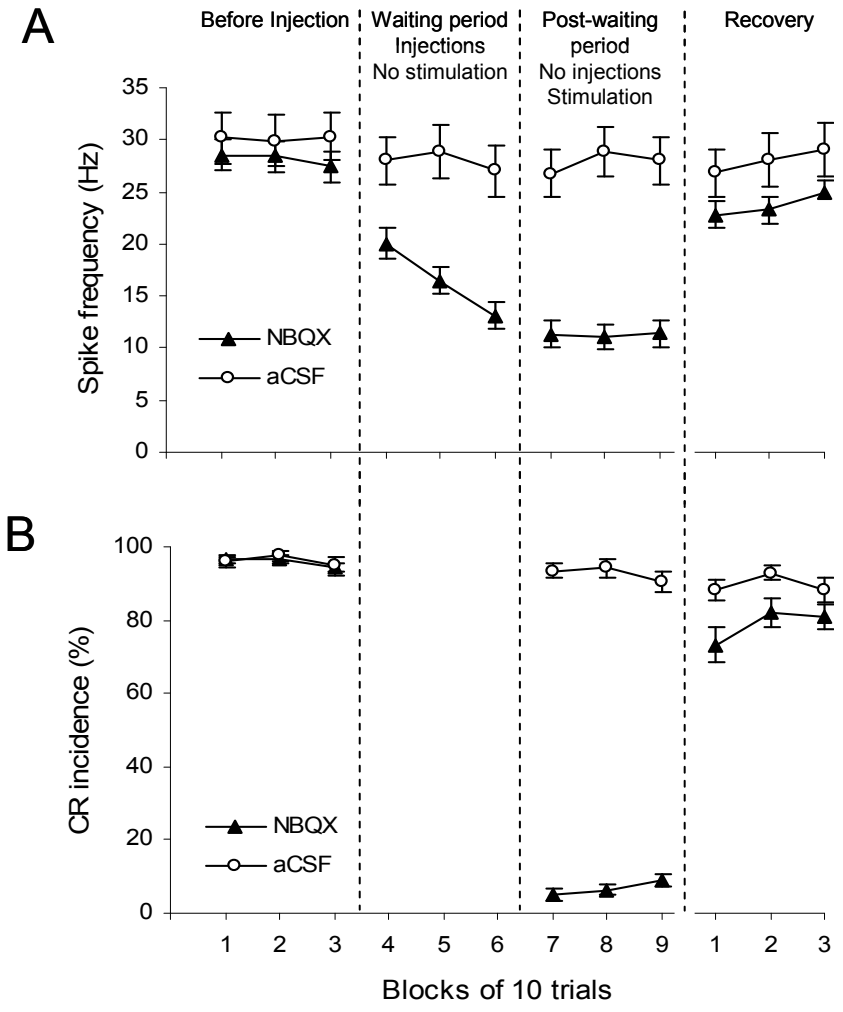


Fig. 8. Group effects of NBQX on CR performance and on IN activity during the waiting test. Each period of the experiment is represented by three consecutive blocks of 10 trials: before injection, no-stimulation waiting period, post-waiting period with stimulation, and recovery. Periods are separated by vertical dashed lines. If the waiting period was longer than 30 trials, then the last 30 trials before stimulation was resumed were included in the plot. *A*: Effects of NBQX (▲) on mean baseline activity (±SE, n=88) of IN cells. The baseline firing rate of IN cells gradually decreased during the waiting period, it remained low when stimulation was resumed and it partially recovered during the last three blocks of the experiment. Injecting aCSF (○) had no significant effect on IN firing rate (n=36). *B*: Effects of NBQX (▲) on mean CR incidence (±SE; n=32 in the same set of experiments as depicted in A. CRs were completely abolished when stimulation was resumed and then partially recovered by the end of testing. This behavioral effect parallels changes in IN activity shown in A. Control injections of vehicle (○) did not affect CR performance.

When stimulation was resumed after the waiting period, CRs were absent at the beginning of the post-waiting test (Fig. 8 B). The mean CR incidence decreased from 94.3±1.3% in the last block of pre-injection trials to 5.0±1.6% during the first 10 post-waiting trials (Blocks 1-3 vs. 7-9, $F_{1,43}$=3165.6, $p<0.001$). Similar to the neuronal activity, partial recovery of CR incidence was observed in the last 30 trials of the recording session (Fig. 8 B), which was significant when compared to post-waiting trials ($F_{1,43}$=341.4, $p<0.001$), but was incomplete relative to the pre-injection level ($F_{1,43}$=24.4, $p<0.001$). The control injections of aCSF did not affect CR incidence (Fig.8 B, Blocks 1-3 vs. 7-9, $F_{1,43}$=3.423, $p=0.071$).

To analyze changes in modulation of IN neurons, all cells from NBQX waiting experiments (n=88) were subdivided into three subgroups based on their pattern of significant modulation in the CS-US interval prior to injections. Our minimum threshold criterion for modulated activity was 2 consecutive 20-ms bins exceeding the upper or lower tolerance limits that were calculated from the first 250 ms of pre-injection peri-stimulus histograms. As a result, sixteen neurons exhibited predominantly excitatory modulation, 12 neurons were largely inhibited and 60 neurons were not modulated. However, 20 of the "non-modulated" cells exhibited a short-latency, short-lasting (20 ms) excitatory response to the CS.

In general, the spontaneous activity of all cell types decreased during the waiting period (Figs. 9B, F and J). When CS-US stimulation was resumed, the modulation of both excitatory and inhibitory cells was also reduced, but not completely abolished (Figs. 9C and G). These changes recovered toward the end of the experiment (Figs. 9D, H, and L). The most affected modulation components were the prolonged excitation and inhibition in the

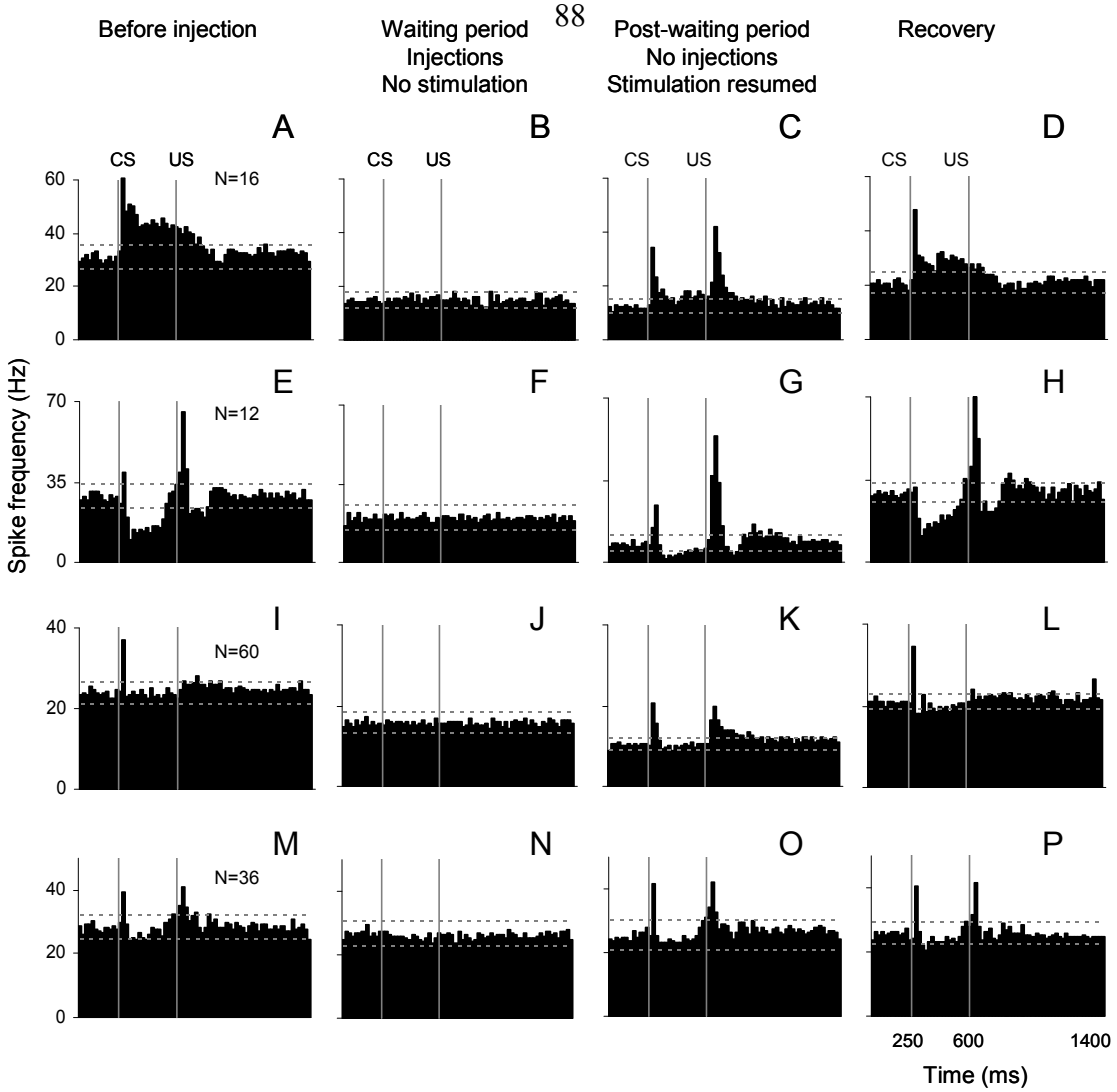


Fig. 9. Effects of NBQX and aCSF injections on cell activity modulation. Mean peri-stimulus histograms are arranged in rows corresponding to cell types and in columns representing the activity before injection, during no stimulation waiting period, post-waiting period when stimulation was resumed, and recovery. *A–D*: cells exhibiting predominantly excitatory responses (n=16). *E-H*: cells that were inhibited during the CS-US interval (n=12). *I-L*: non-modulated cells (n=60). *M–P*: cells recorded during control injections of vehicle (n=36). CS, conditioned stimulus onset; US, unconditioned stimulus onset. Bin width is 20 ms. Horizontal lines in each histogram represent tolerance limits. For more description see section 3.4.

CS-US interval (Fig. 9A vs. 9C and 9E vs. 9G). Interestingly, short-latency excitatory responses to the CS and US proved to be relatively resistant to the NBQX effect. Since the IO has been proposed to provide the intermediate cerebellum with the US information, it was surprising that relative responses to the US were not dramatically reduced. Unexpectedly, the responses to the US became more prominent in all three cell types (Figs. 9C, G and K). In contrast to NBQX experiments, control injections of the vehicle in the IO had no significant effects on baseline activity or modulation (Fig.9M-P).

Since the population super-histograms (Fig. 9) could have means disproportionately affected by the depth of modulation of individual cells, we also constructed binary histograms. By depicting cumulative frequencies of significant excitatory and inhibitory modulation within each 20-ms bin, this plot (Fig. 10) disregards amplitude of modulation by providing information about the percentage of significantly modulated cells. This analysis showed similar results as described above. Specifically, the incidence of prolonged inhibition and excitation in the CS-US interval decreased following the NBQX injection (Figs. 10A vs. B). Interestingly, only inhibitory parts of this response component recovered well by the end of the experiment (Figs. 10B vs. C). Similar to effects seen in Fig. 9, the frequency of short-latency responses to the CS was not reduced in the post-injection test. Remarkably, injecting NBQX in the IO increased the frequency of both short- and long-latency excitatory responses to the US (compare Figs. 10A and B) and this effect recovered by the end of the experiment (Fig. 10C).

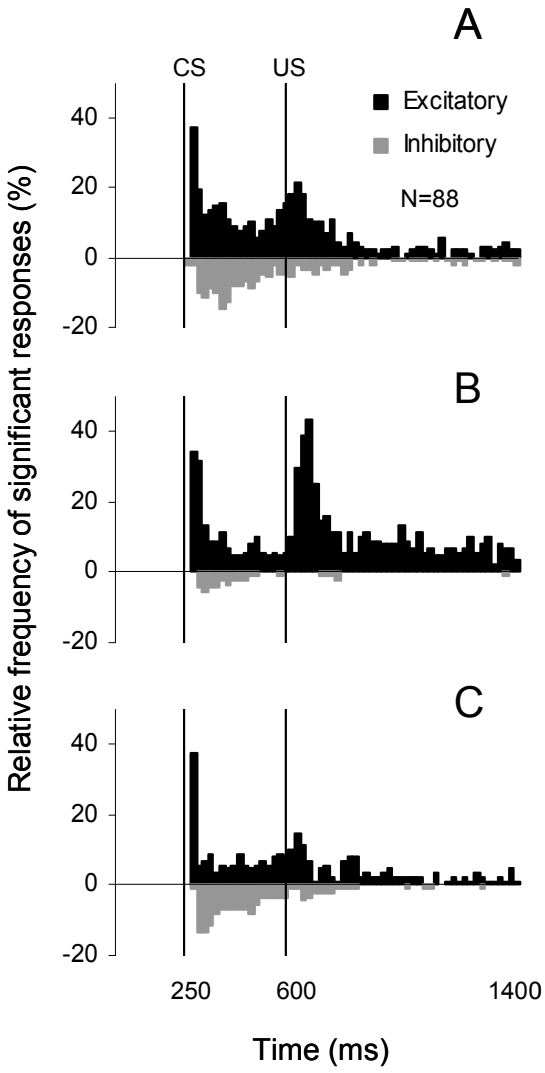


Fig. 10. Frequency histograms of the incidence of significant excitatory (black bars) and inhibitory (gray bars) responses in the population of 88 cells recorded during the NBQX waiting experiments (*A*, before injection; *B*, after injection when stimulation was resumed; *C*: during last 30 trials of experiments). The fraction of cells exhibiting long-latency excitatory or inhibitory responses in the CS-US interval decreased following the NBQX application. Also, the incidence of short-latency excitation to the CS remained unchanged and the incidence of excitatory responses to the US increased following NBQX. These effects tended to recover toward the end of experiments.

3.5 Discussion and Conclusions

The present study demonstrated that injections of the AMPA/kainate receptor antagonist, NBQX, in rostral parts of the inferior olive abolish the expression of eyeblink CRs. This NBQX-induced CR suppression exhibited variably delayed onset timing, ranging from an immediate effect to 35 minutes post-injection. Simultaneous recordings of IN single-unit activity revealed that CR abolition was associated with decreased tonic activity and changes in evoked neuronal responses in deep cerebellar nuclei. The waiting test, in which CS and US presentations were delayed to permit drug diffusion, showed that these effects were simply a function of time and therefore did not require post-injection presentations of stimuli.

3.5.1 Involvement of the IO in expression of CRs – tonic dysfunction vs. cerebellar learning hypothesis

The expression of eyeblink CRs in the rabbit depends critically on the intermediate cerebellum. The IO, which is the exclusive source of cerebellar climbing fibers, is a pivotal component of cerebellar eyeblink-related circuits. A sub-division of the IO, the dorsal accessory olive, receives information about peri-ocular stimulation from sensory trigeminal nuclei (Walberg, 1982; Gellman et al., 1983; Huerta et al., 1983) and transmits this US signal to the intermediate cerebellum. Previous lesion and inactivation studies (Yeo et al., 1986; Welsh and Harvey, 1998) demonstrated that blocking the IO input to the cerebellum abolishes CRs immediately following the experimental intervention. Authors of these behavioral studies speculated that a ***tonic dysfunction of the cerebellar circuit*** could explain this effect. Specifically, they suggested that CR deficits were caused by a non-specific tonic

suppression of activity in cerebellar nuclei that is known to be associated with IO lesions/inactivations (Benedetti et al., 1983; Zbarska et al., 2007). These findings were challenged by reports that lesioning (McCormick et al., 1985) or inactivating (Medina et al., 2002) the IO produces not an immediate, but gradual, extinction-like suppression of CRs. These findings were interpreted in terms of the ***cerebellar learning hypothesis***, which suggests that the IO input is required for the maintenance of memory traces in the cerebellum (Thompson et al., 1998). According to this concept, blocking the IO gradually weakens cerebellar responses to the CS, resulting in CR extinction. Resolving these conflicting inferences from behavioral studies requires measurements of cerebellar neuronal activity during IO manipulations. To address this challenging issue, we applied our method of long-term single-unit recording to experiments involving IO pharmacological manipulations in trained rabbits.

The behavioral component of the present study clearly supports the tonic dysfunction hypothesis. Blocking the IO's AMPA/kainate glutamate receptors with NBQX abolished CRs either almost immediately, or with a short delay. This rate of NBQX-induced CR suppression was appreciably faster than the rate of normal behavioral extinction previously observed in our laboratory (Nilaweera et al., 2005). In the waiting period test, CRs were abolished immediately, starting from the first trial of resumed CS and US presentations. These findings are inconsistent with a cerebellar learning hypothesis which postulates that NBQX-induced CR "extinction" follows the presentation of paired stimuli and should have a rate comparable to normal behavioral extinction (Medina et al., 2002).

Since presentation of stimuli was not required to observe CR abolition, we suggest that some time-dependent process, such as drug diffusion to relevant parts of the IO complex,

explains the delayed cases of the drug effect. The drug diffusion explanation is consistent with the histological reconstruction of our injection sites. First of all, it is unlikely that CR suppression was due to the drug spreading to some other eyeblink-related target outside of the IO (Hiraoka and Shimamura, 1977; Holstege, 1990). Injections of NBQX dorsal to the IO made prior to the last mapping experiment had no effect on CRs. Also, the fastest effects were observed in animals with injection sites closest to the rostral-medial pole of the DAO, which is known to receive somatosensory information from the contralateral facial region (Gellman et al., 1983).

The tonic dysfunction hypothesis is strongly supported by our recordings of IN single-unit activity. The gradual reduction of CRs is well correlated with a gradual inhibition of the spontaneous firing rate of IN neurons in immediate drug tests. The waiting experiments clearly dissociated these processes because the IN firing rate declined during the waiting period when rabbits were not stimulated. When the stimulation resumed, CRs were immediately absent on the background of already suppressed IN activity. This stimulation-independent and NBQX-induced suppression of IN firing rate is consistent with previous reports of the tonic effects of blocking IO input to the cerebellum (Benedetti et al., 1983; Bengtsson et al., 2004). It appears that NBQX down-regulated the IO's firing rate and this up-regulated the activity of Purkinje cells in the cerebellar cortex (Batini et al., 1985; Lang, 2001; Bengtsson et al., 2004). The increased activity of these inhibitory cells then suppressed activity of IN neurons, as shown by our measurements. Since sustained inhibition of IN neurons suppresses CR expression (Aksenov et al., 2004), we presume that the NBQX-triggered tonic shifts in the activity of cerebellar neurons are either partly or completely responsible for the observed CR deficit. Interestingly, even though the NBQX-

induced suppression of IN activity was sufficient to prevent CR expression, it was smaller than what we reported in DGG injection experiments (Zbarska et al., 2007). Since DGG blocks AMPA/kainate and also NMDA receptors, it appears that NMDA receptors participate in the regulation of IO firing rates (Placantonakis and Welsh, 2001).

Although the tonic activity of some IN neurons was completely suppressed, most cells continued to fire but at significantly decreased rates. It is not clear whether the residual presence of tonic modulated activity in the IN is related to an incomplete block of AMPA/kainate receptors in areas of the IO that project to our recording sites. Exploring this scenario would require injecting excess amounts of NBQX. We have not yet performed this test because interpreting the results of a more complete block could be confounded by excess drug spreading to other parts of eyeblink circuits, which would have required additional controls. Similar to changes in spontaneous firing rates, the depth of response modulation and its incidence were reduced, but not completely eliminated. The most reduced response components were longer-latency (>60 ms) excitatory and inhibitory responses in the CS-US interval. On the other hand, short-latency responses to the CS remained relatively stable and the incidence of responses to the US actually increased. The increased responses to the US are surprising since the IO is presumed to be a major source for the US signal. It is likely that after IO inactivation with NBQX, information about the US reached the cerebellum through trigemino-cerebellar mossy fiber projections that are known to convey somatosensory information from facial areas (Woolston et al., 1981; Kassel et al., 1984; Ikeda and Matsushita, 1992). This finding raises the possibility of functionally significant, extra-olivary US inputs to eyeblink-related IN neurons that may need to be incorporated in the models of eyeblink conditioning.

In summary, we propose that the NBQX-induced abolition of CRs was most likely caused by the stimulus-independent suppression of IN's tonic rate and of its neuronal responses in the CS-US interval. This conclusion contradicts Medina et al. (2002), who reported stimulation-dependent CR extinction during continuous injections of NBQX in the IO. We could not replicate this result in spite of using the same drug concentration and a very similar experimental setup. On the contrary, smaller amounts of NBQX in a larger group of rabbits invariably abolished CRs in a stimulation-independent fashion. In one of our animals, a much smaller amount of NBQX (eight-times smaller concentration) also yielded CR abolition. Two aspects of Medina et al.'s study could have contributed to this difference. First of all, their study had a complex design with animals being subjected to infusions of picrotoxin and vehicle and to repeated extinction and CR re-acquisition sessions prior to their NBQX tests. Although unlikely, it can not be excluded that these procedures altered the physiology of underlying circuits in a manner making them resistant to NBQX effects on cerebellar tonic activity. Another possibility is that repeated injections in Medina et al.'s study induced the formation of glial scarring that progressively degraded the effective drug diffusion, yielding more delayed effects in their waiting test. This is a common problem in studies that involve repeated injections at the same site. Anticipating this effect, we added an additional five minutes to the waiting period test in the present study. However, independent of the resolution of this conflict, our data demonstrate that NBQX injected in the IO abolishes CRs not because of unlearning, but by rendering cerebellar circuits dysfunctional.

3.5.2 Implications for future studies

Although this study supports the tonic dysfunction hypothesis and challenges certain tenets of the cerebellar learning hypothesis, it should be emphasized that these hypotheses are not mutually exclusive. The IO possibly could provide the intermediate cerebellum with the "teaching" US input as postulated in the cerebellar learning hypothesis while at the same time regulating tonic activity in the cerebellum. This possible dual role of the IO has important consequences for developing experimental strategies in addressing IO participation in eyeblink conditioning. New methods will have to be developed to block IO responses to the US without significantly affecting its spontaneous firing rate. The important contribution of this study is its demonstration that blocking AMPA/kainate receptors in the IO does not achieve this desirable effect. An equally important implication is that the use of electrophysiological recordings performed simultaneously with pharmacological manipulations and behavioral assessment of CRs essential for deciphering the mechanism responsible for changes observed following cerebellar manipulations.

3.6 Acknowledgements

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CHAPTER 4. ULTIMATE TEST OF IO FUNCTION: BLOKING IO INPUTS WHILE MAINTANING ITS TONIC ACTIVITY

4.1 Abstract

The inferior olive is a major component of the eyeblink conditioning neural network. According to the cerebellar learning hypothesis, the IO functions in supplying the cerebellum with a "teaching" unconditioned stimulus input required for the acquisition of conditioned responses, and predicts that blocking this input should lead to the extinction of CRs.

Testing this hypothesis requires blocking sensory inputs to the IO without affecting its tonic discharge. Since blocking sensory inputs to the IO with glutamate antagonists reduces its firing rate, new approaches have to be developed to counter-balance these tonic drug effects. In the present study we examined two drug candidates, harmaline and gabazine, for their potential to elevate the IO's spontaneous activity.

We found that systemic administration of harmaline abolished CRs and induced body tremor. Surprisingly, intra-olivary harmaline administration had no effect on CRs, indicating that the systemic harmaline effect was not mediated through the inferior olive. In contrast to harmaline, the GABA-A receptor antagonist gabazine injected in the IO increased tonic eyelid closure, shortened CR onset latency and increased neuronal activity in the IN. These observations indicate that gabazine increased IO firing rate and that it could be potentially used to offset tonic effects of glutamate drugs on IO activity. To assess whether gabazine indeed has this property, we injected the IO of trained rabbits first with the glutamate antagonist γ-glutamylglycine (DGG) and then with gabazine. We found that gabazine

recovered CRs abolished with DGG. Electrophysiological recordings revealed that behavioral recovery of CRs was paralleled by the recovery of neuronal activity in the IN. These findings suggest that the level of IO neuronal excitability is critical for CR performance and that maintaining excitability at near normal levels may be more important for the maintenance of CRs than are the US signals they carry to the cerebellum.

4.2 Introduction

The inferior olive is an important part of eyeblink conditioning circuits. Inactivating or lesioning the IO blocks the expression and acquisition of CRs in the rabbit, but the mechanisms of this phenomenon are not known. The cerebellar learning hypothesis suggests that inactivating the IO deprives the cerebellum of the teaching US signal and this translates into a gradual CR abolition interpreted as behavioral extinction. Our previous results revealed that blocking IO activity abolishes CRs by producing a tonic dysfunction of cerebellar circuits (Zbarska et al., 2007). Based on these data we concluded that the IO plays a tonic regulatory role in the neuronal circuit involved in classical conditioning. This conclusion, however, did not disprove the cerebellar hypothesis. This is because the tonic consequences of IO manipulation may have obscured the IO's participation in learning. As we indicated in our literature review, an ultimate test of the cerebellar learning hypothesis would require blocking sensory inputs to the IO without affecting its spontaneous tonic activity.

Somatosensory information from the face reaches the IO through trigeminal inputs, which are known to be glutamatergic (van Ham and Yeo, 1992; Lang, 2001). We have confirmed Lang's (2001) finding that blocking glutamate has adverse side-effects in the form

of suppressed IO tonic activity (Zbarska et al., 2007). Clearly, blocking sensory inputs to the IO to test their role in learning requires carefully calibrated methods for blocking only these inputs without also causing major shifts in IO intrinsic activity. To accomplish this, we have been developing a new multi-injection protocol in which injections of a glutamate antagonist are followed by injections of another drug that would restore tonic activity in the IO.

We tested two potential drug candidates - harmaline and gabazine – for restoring tonic activity. Harmaline is an alkaloid whose systemic administration is known to produce synchronized rhythmic activation of IO neurons and to induce a strong body tremor (Lamarre and Weiss, 1973; Llinas and Volkind, 1973; Headley et al., 1976). Also, systemic administration of harmaline abolishes CR expression in the rabbit, and it has been speculated that this effect is mediated by the IO (Turker and Miles, 1984; Harvey and Romano, 1993). De Montigny and Lamarre (1973) recorded from the IO during systemic harmaline injections, which revealed synchronized rhythmic activation in the caudal half of the medial dorsal accessory IO. Local application of harmaline into the IO of decerebrated cats produced similar results, characterized by sustained rhythmic activity in the caudal halves of the inferior olive. These authors confirmed that harmaline most likely acts directly on the inferior olive to produce body tremor (De Montigny and Lamarre, 1975). Because of possible species-specific differences in the effects of harmaline (Miwa et al., 2006), we first compared effects of systemic and intraolivary injections on CR expression. We found that harmaline is not suitable for restoring tonic activity because its effects on CRs are not mediated by the IO.

In a second group of experiments we tested the GABA-A receptor antagonist gabazine, which activates the IO by blocking its inhibition (Ueno et al., 1997). It is known

that the IO receives GABA-ergic projections from deep cerebellar nuclei (Fredette and Mugnaini, 1991; Ruigrok and Voogd, 1995; Svensson et al., 2005) and the local application of the GABA-A receptor antagonist picrotoxin increases tonic activity in the IO (Lang, 2002). Our exploratory experiments revealed that the intraolivary injection of gabazine does not affect CR incidence, but it does shorten CR latency and increases the baseline activity of IN cells. These preliminary results show that because gabazine increases neuronal activity in the IO, it thus has the potential for restoring tonic activity suppressed by glutamate antagonists.

To assess this issue, we injected the IO in trained rabbits first with DGG and then with gabazine. The results of this dual micro-injection experiment indicate that gabazine accelerates the recovery of CRs abolished by DGG. These behavioral results were supported by pilot electrophysiological recordings showing that the recovery of DGG-suppressed neuronal activity in the IN could also be accelerated by gabazine injections.

4.3 Materials and Methods.

4.3.1 Subjects

To explore the effect on CRs of intracranial injections of harmaline, gabazine and their combination with DGG, we implanted four male, New Zealand White Rabbits (Harlan, Indianapolis, Indiana), weighing 2.5–3.0 kg (3–4 months old at the beginning of experiments) with injection cannulae in the IO. To record the effect of IO injections on neuronal activity in the IN, one of these rabbits was also equipped with a microelectrode assembly in the IN for electrophysiological recording of unitary activity. Rabbits were housed individually on a 12/12 hr light/dark cycle and provided with food and water *ad libitum*. All experiments were performed in accordance with the National Institutes of Health's "Principles of Laboratory Animal Care" (publication No. 86-23, revised 1985), the

American Physiological Society's "Guiding Principles in the Care and Use of Animals," and the protocol approved by Iowa State University's Committee on Animal Care.

4.3.2 Surgery

After exposing the skull, three stainless steel anchor screws and a 28-gauge injection guide tube were implanted. For targeting anterior parts of the right IO, the following stereotaxic coordinates were used: AP = x - (0.69x + 4.5) mm anterior to lambda (x is horizontal distance between bregma and lambda in mm), ML = 1.0 mm lateral to lambda, and DV = 22.4 mm ventral to lambda. The patency of the injection guide tube was protected between experiments by a 33-gauge stainless steel stylet.

One rabbit was implanted with a microelectrode array/micromanipulator assembly targeting the left IN as in (Aksenov et al., 2004). The manipulator contained three bundles of microwire electrodes totaling 14 electrodes (stainless steel, 18 μm diameter, Formvar insulation). The microwires were connected to a 15-pin connector embedded in dental acrylic. The stereotaxic coordinates for the IN implant were: AP = x - (0.69x + 4.8) mm anterior to lambda (x is distance between bregma and lambda in mm), ML = 5.0 mm lateral to lambda, and DV = 14 mm ventral to lambda. The guide tube, manipulator, connector, anchor screws, and a small Delrin block designed to accommodate an air-puff delivery nozzle and an eyeblink sensor were secured in place with dental acrylic. Following surgery, the manipulator and injection guide tube were protected with removable Delrin caps. All animals were treated with antibiotics during a 5-day post-surgical recovery period.

4.3.3 Training procedures

One week post-surgery, all rabbits were first adapted to a restraint box in a sound-attenuated experiment chamber for 3 days, 30 min per day. Head movements were not restricted either during adaptation or during experiments. Box-adapted animals were conditioned in the standard delay classical conditioning paradigm until they reached 90% of conditioned responses (CRs) for at least two consecutive days. The conditioned stimulus (CS) was a binaural 450-ms, 80-dB SPL, 1-kHz tone, superimposed on a 70-dB white noise. As the unconditioned stimulus (US), a 100-ms, 50-psi (at the source) air puff was applied to the left eye. The inter-stimulus interval was 350 ms. The inter-trial interval varied pseudo-randomly from 15 to 25 s. Rabbits had one conditioning session of 100 paired trials per day until they reached 90% of CRs for two consecutive days.

4.3.4 Injection procedures

Before each injection experiment a 33-gauge stainless steel needle was inserted in the intracranial guide tube. The injection needle was connected to a 10-µl Hamilton syringe using transparent Tygon tubing. The pressure microinjections were performed manually at a rate of 0.5 µl/min. The injected volume was monitored by observing the movement of a small air bubble relative to gradation marks on the transparent tubing. During injection sessions, 40 trials of paired stimuli were applied prior to the injection to assure that needle insertion had no effect on CR performance. Injections were not administered if rabbits had less than 85% CRs during the pre-injection baseline period. NBQX (Sigma-Aldrich) was dissolved in artificial cerebrospinal fluid (aCSF, Medina et al., 2002) and its pH was adjusted to 7.4±0.1.

Functional mapping. Since previous studies have shown that AMPA receptor antagonists decrease neuronal activity in the IO (Lang, 2002) and abolish eyeblink CRs (Zbarska et al., 2007) we used 1.0-µl injections of the AMPA/kainate and NMDA receptor antagonist, DGG (Tocris Bioscience, 100 nmol in 1µl), for functional mapping. During the mapping stage, the first exploratory DGG injection was performed at the depth of the guide tube tip. Mapping sessions consisted of 40 pre-injection trials and 80 post-injection trials, which were administered immediately following the initial DGG injection. If no effect on CR incidence was observed, the depth of injection was adjusted, progressing 0.5 mm deeper the next day. This daily increase of injection depth was repeated until DGG completely abolished CRs, or until the needle reached the base of the skull.

Intracranial injections of harmaline and gabazine. After an effective injection site was established, the separate effects of 1.0 µl of harmaline (15 g/L) and 1.0 µl of gabazine (0.75g/L) in the IO on CR expression were tested. Sessions at this stage consisted of 40 pre-injection trials and 80 post-injection trials, which were administered without delay immediately following the injection. All drugs were dissolved in aCSF (Medina et al., 2002) and their pH was adjusted to 7.4±0.1. Only one drug was injected on any given experiment day. During vehicle control experiments, 1.0 µl of aCSF was administered into the IO using the same technique as in drug experiments.

Injections during combination experiments. After successful gabazine-alone effects had been established, a combination experiment was conducted. These combination experiments tested whether CRs abolished by DGG can be restored by increasing IO tonic activity. To block glutamate input to the IO, 1.0 µl of DGG (20 g/L) was injected just as during functional mapping. Once DGG abolished CRs for at least 20 consecutive trials, the

experiment was paused momentarily to replace the injection needle for DGG with the injection needle for gabazine. As soon as paired stimulation was resumed, 1.0 µl of gabazine (0.75 mg/ml) was injected to the same site at the rate of 0.5 µl/min every 40 trials without pausing stimulation until a change in behavior was observed, or for an additional 120 trials if no effects were evident. Vehicle control experiments utilized aCSF instead of gabazine.

Systemic administration of harmaline. Previous studies reported that systemic administration of the alkaloid harmaline suppressed CR expression and produced body tremor (Llinas and Volkind, 1973;Turker and Miles, 1984). To verify these findings in our preparation, the same four animals examined in the intracranial injection experiments were also tested following the systemic administration of harmaline. In these experiments, 3.0 ml of harmaline (15 g/L) were subcutaneously administered after 40 pre-injection trials. In vehicle control experiments, 3.0 ml of saline were administered subcutaneously.

4.3.5 Data recording and analysis

During experimental sessions the eyeblinks were recorded using a wide field-of-view infrared sensor (Ryan et al., 2006). The sensor's signal was amplified, digitized at 1 kHz with 12-bit resolution, and displayed and stored on a custom data acquisition system. Data were acquired for 1400 ms in each trial, starting 250 ms before the CS onset. Eyeblink responses beginning between 80 ms after CS onset and up to the US onset were considered CRs when they exceeded the mean of the signal in the 250-ms baseline period by more than five standard deviations (approximately 0.1 mm). Spontaneous responses were defined as trials in which the difference between the maximum and minimum values in the baseline period exceeded 0.5 mm. Any blink exceeding the CR threshold within 80 ms after CS onset

was recognized as an alpha response. Trials containing spontaneous blinks or rare alpha responses were stored but were not included in further data analyses. Measurements of eyeblink responses were used for calculations of CR incidence per block of 10 trials in each session.

In addition to the visualization of eyeblink traces on the computer monitor, two video cameras were installed in the experiment chamber to monitor general animal behavior during experiments. One camera provided a front view of the rabbit's head and the other one, configured for infrared viewing, was positioned on the side of the trained eye. This setup proved to be invaluable for monitoring rabbit behavior continuously.

To monitor movements of the rabbit's head during harmaline-induced tremor, an accelerometer was attached to the head stage. Recordings from the accelerometer were displayed and stored in 1400-ms epochs corresponding to individual trials.

In the rabbit implanted with microelectrodes, multiple single-unit signals were fed through a custom miniature 14-channel FET-based preamplifier to a multi-channel differential amplifier system (Grass-Telefactor model 12 Neurodata System). The amplified and band pass-filtered (300 Hz-3 kHz) signal was digitized (25 kHz/channel) using a custom data acquisition system, and was displayed and stored in 1400-ms epochs corresponding to individual trials. Unit discrimination was performed offline using threshold detection followed by a cluster analysis of scatter plots of time and amplitude distances between the peak and valley of individual action potential waveforms. The discriminated data were processed using custom software in addition to a commercial data analysis package (NeuroExplorer, Nex Technologies). Cross-correlation analysis was performed for each unit to eliminate multiple inclusions of the same unit recorded on different wires within a bundle.

Raster and peri-event histograms were constructed for each unit. For cells recorded during gabazine-alone experiments, separate histograms were constructed for 40 pre-injection trials and all post-injection trials. For cells recorded during combination experiments, separate histograms were constructed for 40 pre-injection trials, for 40 trials of complete DGG-induced CR abolition and for 40 trials of complete gabazine-induced CR recovery. All group data are reported as mean ± standard error of the mean.

4.3.6 Histology

After all experiments were completed, rabbits were deeply anesthetized with a mixture of ketamine (100 mg/kg), xylazine (12 mg/kg), and acepromazine (3 mg/kg). The injection site was marked by injecting 1 μl of tissue-marking dye. Anesthetized animals were transcardially perfused with 1 L of phosphate-buffered saline followed by 1 L of fixative (10% buffered formalin) and 1 L 10% potassium ferrocyanide in 10% formalin. The potassium ferrocyanide was applied only to the IN-recording rabbit, where the location of electrodes was marked by passing 10 μA anodal DC current through each wire for 20 s. Brains were removed and placed in a solution of 30% sucrose and 10% formalin. After one week, brains were sectioned coronally at 50 μm on a freezing microtome. Sections were mounted onto gelatin-coated slides, dried, and stained with Luxol blue and neutral red, except for the IN recording rabbit, whose IN sections were stained with ferrocyanide hydrochloride and neutral red. Locations of injection sites were determined using bright field microscopy and transferred to a standardized set of coronal sections of the rabbit medulla. Locations of electrode marks were transferred to a standardized set of coronal sections of the rabbit cerebellar nuclear region.

4.4 Results

4.4.1 Location of injection sites and general observations

Four rabbits were implanted with an injection cannula in the IO. One of them also had recording electrodes implanted in the IN for electrophysiological recording. The histological reconstruction of IO injection sites revealed that they were located in the rostral portion of the inferior olivary complex, close to the rostral part of the dorsal accessory inferior olive (Fig.1).

All rabbits reached the 2-day 90% CR criterion in 8 to 15 training sessions. Once effective injection sites were established through functional mapping using DGG, intracranial effects of harmaline and gabazine on CR expression were tested in separate experiments. Unexpectedly, microinjections of harmaline in the IO did not affect CR expression and had no visible behavioral side effects. On the other hand, injections of gabazine at identical injection sites shortened CR latency (Fig. 2B), produced tonic eyelid closure (Fig. 2A) and induced head rotation to the right and a postural asymmetry characterized by limb extension on the left side of the body once released from the restraint box.

Since injections of harmaline in the IO had no effect on CRs, its effectiveness was tested systemically by injecting it subcutaneously in the same subjects. In agreement with previous studies, subcutaneous injections of harmaline suppressed CR performance (Fig. 3) and produced 8-11 Hz head/neck tremor as documented by the accelerometer measurements. This tremor, however, did not propagate to the eyelid effector system.

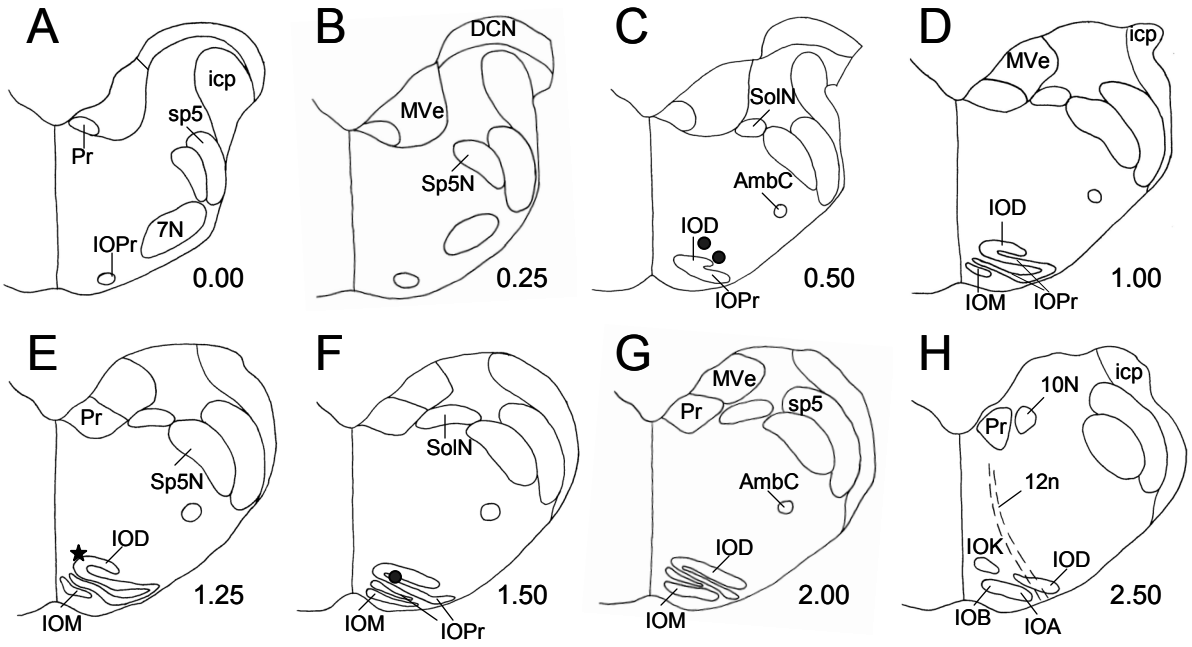


Fig. 1. Location of injection sites in the IO in the rabbits implanted with an injection cannula only (●, n=3) and with an injection cannula and microelectrode assembly (★). The identified sites in individual animals were transferred to a set of standardized coronal sections of the rabbit medulla, arranged in rostral-to-caudal order (*A*-*H*). All injection sites were found within or close to the rostral part of the inferior olivary complex. Injections of DGG at all marked sites abolished CR expression. The anterior-posterior distance of each section from the most rostral pole of the IO in mm is labeled on the lower right side of individual sections. IOD - dorsal accessory inferior olive; IOM - medial inferior olive; IOPr - principal inferior olive; IOA - subnucleus A of the medial inferior olive; IOB - subnucleus B of the medial inferior olive; IOK - cap of Kooy of the medial inferior olive; Sp5N - spinal trigeminal nucleus; sp5 - spinal trigeminal tract; Pr - prepositus hypoglossal nucleus; 7N - facial nucleus; icp - inferior cerebellar peduncle; MVe - medial vestibular nucleus; SolN - solitary nucleus; AmbC - ambiguous nucleus; 12n - hypoglossal nerve; 10N - dorsal motor nucleus of the vagus; DCN – dorsal cochlear nucleus.

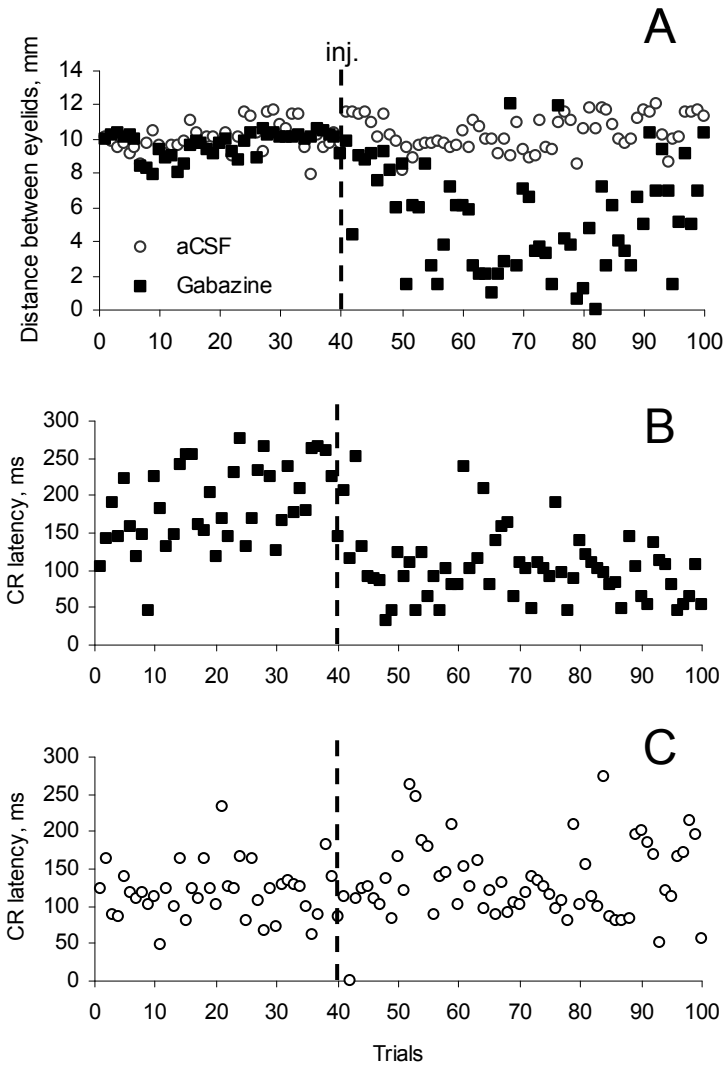


Fig. 2. An example from one subject of the effects of IO injections of gabazine and vehicle (aCSF) on eyelid position and CR latency. *A:* The eyelid aperture before and after gabazine and vehicle injections in the IO. Gabazine produced tonic eyelid closure while aCSF did not affect eyelid position. *B:* The latency of CRs was shortened after gabazine injections in the IO. *C:* Microinjection of vehicle had no effect on CR latency.

4.4.2 Effect of harmaline on CR expression

Effect of intracranial harmaline injections. Injecting 1.0 µl of harmaline in the IO did not affect CR expression (Figs. 3B, 4). At the group level, CR incidence after injecting harmaline into the IO (90.9±4.8%) was indistinguishable from the vehicle control (90.5±6.7%). The effectiveness of each rabbit's injection site had been verified in prior experiments whereby inactivating the IO with DGG suppressed CRs when compared with the pre-injection performance (Figs. 3A, 4). To examine whether the lack of harmaline effects could be related to degradation of the injection site, in the following session all rabbits were injected with DGG again (Figs. 3C, 4). In all rabbits the injection site was still effective since DGG reduced CR incidence from 92.5±7.5% during the last pre-injection 10-trial block to 7.5±4.8% during the fourth post-injection 10-trials block. Vehicle control injections at the same injection site did not have any effect on CR expression (Figs. 3D, 4).

Effect of systemic harmaline injections. The absence of an expected harmaline effect could implicate the quality of the drug. To verify its potency, we conducted a systemic injection bioassay in the same set of animals. In agreement with previous reports (Turker and Miles, 1986), subcutaneous injection of 3.0 ml harmaline (15 mg/kg) gradually abolished CR expression (Fig 5A). This effect was usually incomplete with infrequent CRs still randomly occurring during the post-injection period (Fig. 5A). At the group level, systemic administration of harmaline decreased CR incidence from 96.1±0.9% pre-injection to 12.9±4.1% in the fifth post-injection block of trials (Fig. 4). Subcutaneous injections of the vehicle control had no effect on CR performance (Fig. 4, 5B).

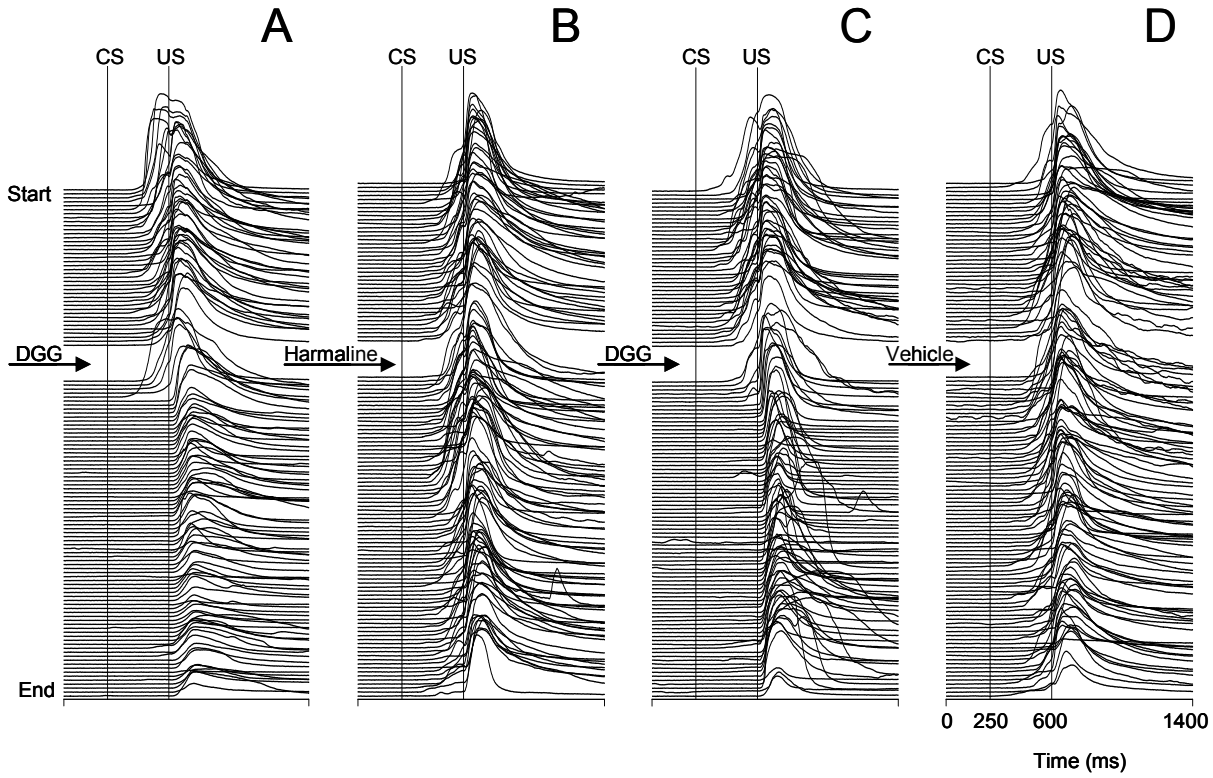


Fig. 3. Four separate experiments from the same animal on the behavioral effects of intracranial injections of harmaline and vehicle (aCSF) at the IO site where DGG abolished CRs. *A, C*: Eyeblinks from an experiment testing the effectiveness of the injection site by microinjecting 1.0 µl of DGG. These tests were performed before and after the harmaline experiment. DGG gradually abolished CRs within 5 minutes of post-injection training, verifying an effective injection site. *B*: Microinjecting 1.0 µl harmaline (15 mg/ml) did not suppress CR performance. *D*: An intracranial injection of the vehicle control (aCSF) did not affect CR performance.

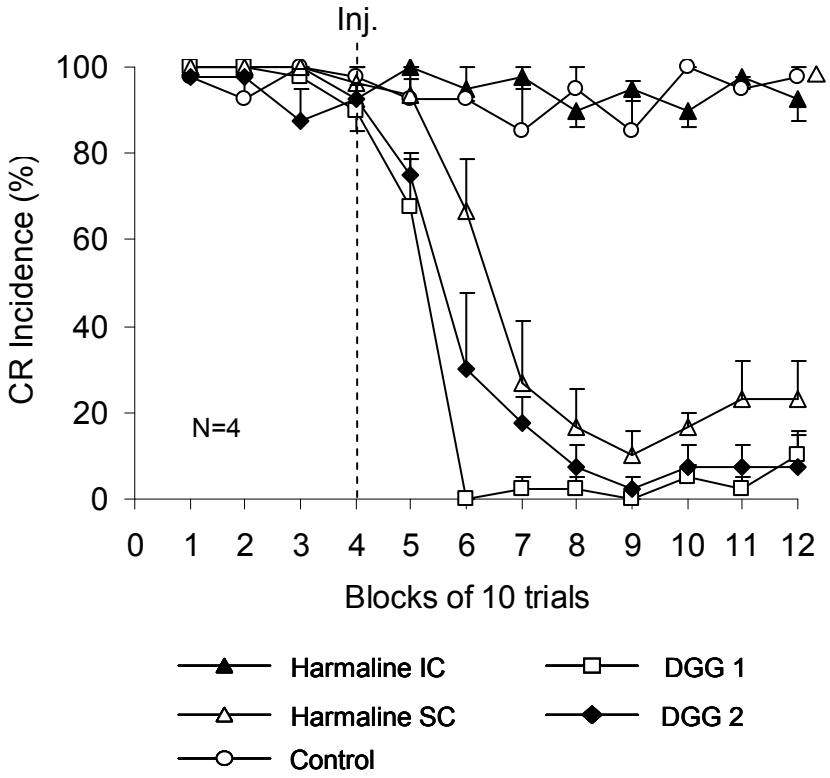


Fig. 4. Group effects of subcutaneous and intracranial injections of harmaline, two DGG injections and vehicle on CR incidence (±SE). Intracranial injections of harmaline and aCSF had no effect on CR incidence. On the other hand, both DGG and systemic harmaline microinjections produced dramatic CR deficits.

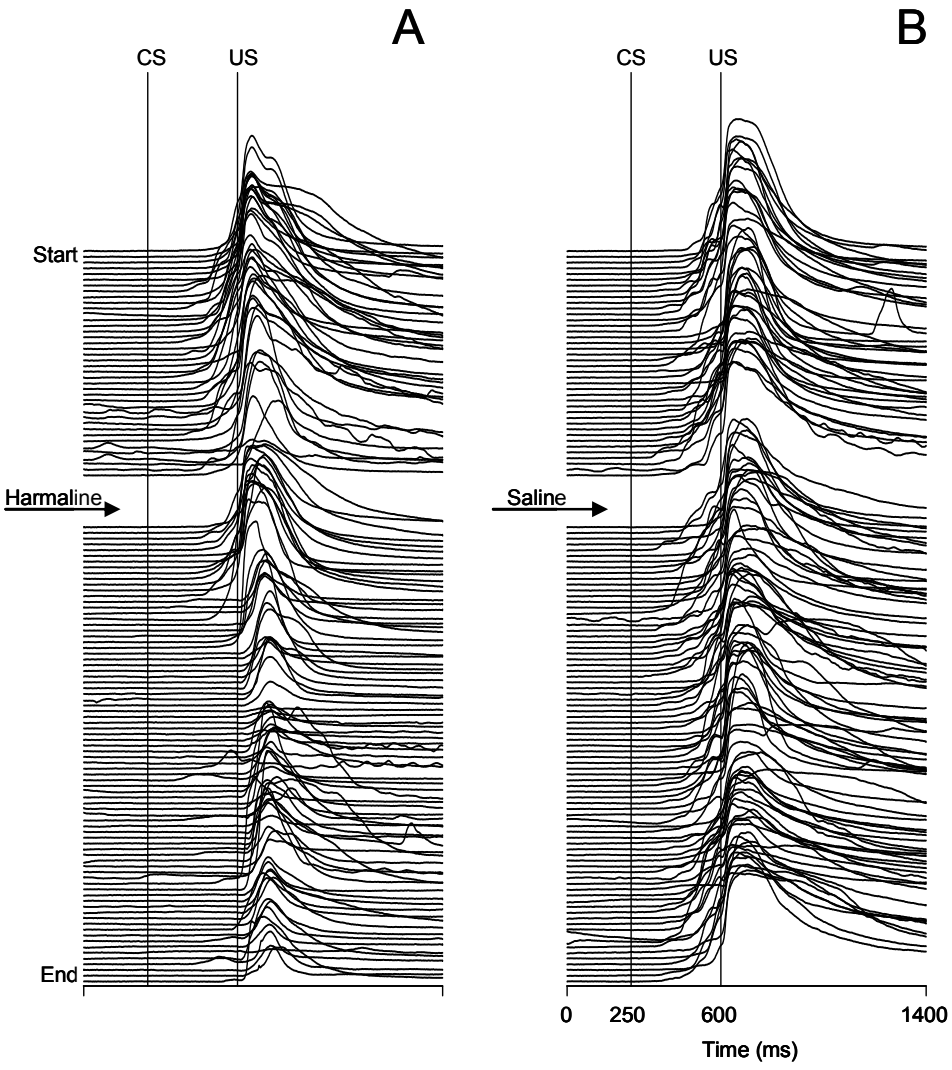


Fig. 5. Individual examples of behavioral effects of subcutaneously injecting harmaline and vehicle (saline) in the same subject. *A*: After the subcutaneous injection of harmaline (15 mg/kg), eyeblink CRs were gradually abolished within 10 minutes of post-injection training. *B*: The subcutaneous injection of saline did not suppress CR performance.

4.4.3 Changes in CR performance and IN neuronal activity after injecting gabazine into the IO

Since intracranial injections of harmaline did not produce expected effects on CR performance, we ran pilot experiments to test a second drug – gabazine – for its potential to elevate tonic activity in the IO. At the IO injection site where DGG had completely abolished CRs (Fig. 6B), 1.0 µl of gabazine (0.75 g/L) did not affect CR incidence (Fig. 6A), but it did shorten CR latency from 184.2±9.0 ms in pre-injection trials to 109.3±10.1 ms in post-injection trials (Fig. 2B, 6A).

Similar behavioral effects of gabazine were observed on another rabbit that had been implanted with an injection cannula in the IO and a microelectrode assembly in the IN (Fig. 7A, B). During that representative experiment, dysinhibiting the IO with gabazine increased the baseline firing rate of an IN neuron (Fig. 7 B, C). This coincided with the shortening of CR latency (Fig.7 B, bold markers). In the unit shown in Fig. 7, baseline activity more than doubled from 6.2±1.0 Hz during pre-injection trials to 14.4±0.4 Hz during 160 post-injection trials. Gabazine also altered the neuronal response to the CS and US (compare Figs. 7 D and E). These data are consistent with the notion of gabazine increasing IO tonic activity, and they suggest that this drug could potentially be used to restore its spontaneous firing rate in experiments blocking glutamatergic inputs to the IO.

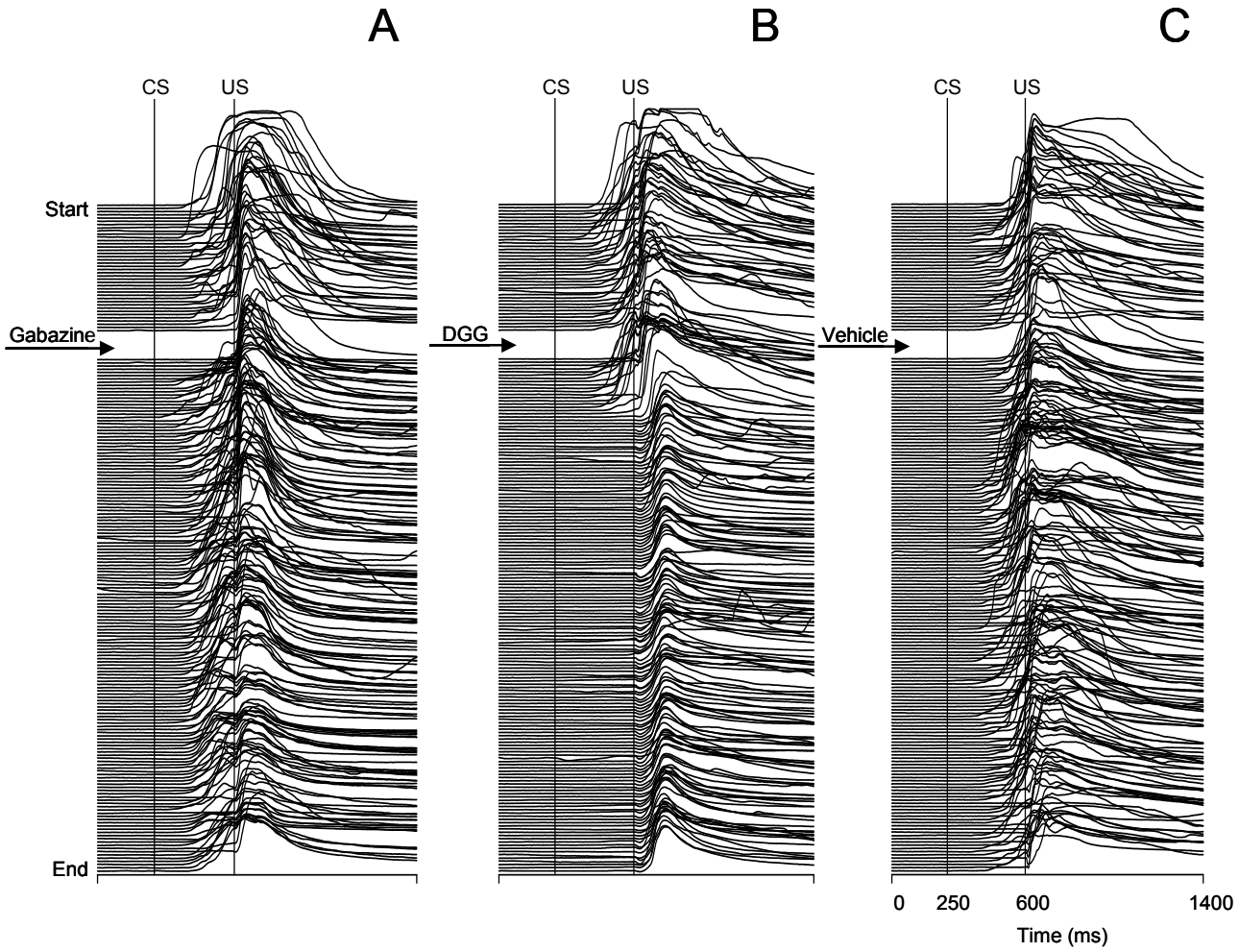


Fig.6. Stack plots of all eyeblinks recorded in three separate experiments in the same animal showing effects of gabazine, DGG and vehicle (aCSF) on CR performance. *A*: Eyeblinks in an experiment where 1.0 µl of gabazine injected in the IO increased the amplitude and shortened the latency of CRs. *B*: Eyeblinks in an experiment where injecting 1.0 µl of DGG in the same place quickly abolished CRs. *C*: Eyeblinks in the control experiment in which 1.0 µl of vehicle (aCSF) did not affect CRs.

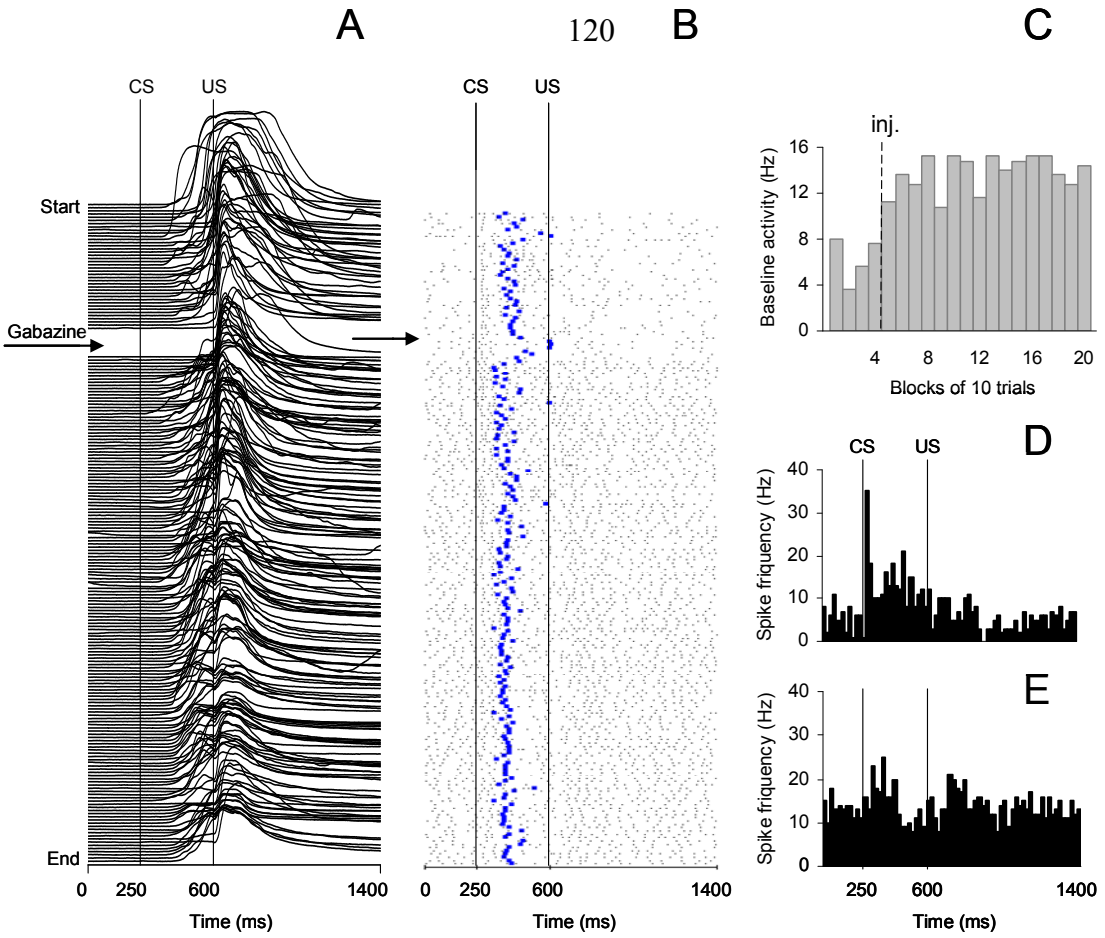


Fig. 7. An example of the parallel effects of gabazine on CR performance and IN cell activity. This experiment consisted of 200 trials with the injection administered after 40 trials. *A*: Stack plot of all eyeblinks before and after gabazine was injected into the IO revealing a shortened CR latency. *B*: Raster plot of action potentials of a single IN unit during the same experiment. The experiment starts at the top with each row representing one trial, and each dot marking the occurrence of an action potential. Coinciding with the shortening of CR latency (the CR onset is labeled with a bold dot in each row), the firing rate of this cell's activity increased as evidenced by the increased density of action potential markers. *C*: Bar plot of the same cell showing that baseline spike frequency nearly doubled after gabazine was injected into the IO. *D:* A peri-stimulus histogram of the same IN unit constructed from 40 pre-injection trials reveals an excitatory response during the CS-US interval followed by post-US inhibition. *E*: Peri-stimulus histogram of the same cell constructed from the first 40 post-injection trials, showing an increased baseline firing rate. Bin width for histograms in *D* and *E* is 20 ms. CS, onset of conditioned stimulus; US, onset of unconditioned stimulus.

4.4.4. Effects of sequential injections of DGG and gabazine into the IO on CR expression and on neuronal activity in the IN

After inferring that gabazine can increase baseline neuronal activity in the IO (see section 3.4.3), we tested whether this drug could be used to counterbalance the tonic effects of glutamate antagonism. In this experiment (see Fig. 8 for an example), glutamate input to the IO was blocked by injecting 1.0 µl of DGG. Once CRs were abolished, two 1.0-µl gabazine injections were administered 40 trials apart at the same injection site (Fig. 8A). This led to a complete recovery of CRs 20 minutes after the first gabazine injection. The size and shape of these recovered CRs were comparable to pre-injection CRs (Fig. 8A). In the control experiment, DGG was followed by vehicle injections administered according to the same schedule as for gabazine in the preceding experiment. Vehicle injections did not accelerate CR recovery, which emerged spontaneously much later toward the end of the experiment (Fig. 8 B).

Single-unit recordings during this experiment revealed that the DGG-induced CR abolition coincided with suppression of the spontaneous firing rate of IN cells (Figs. 9A, B, C). While CRs were abolished and baseline activity of the illustrated unit was suppressed, a strong excitatory response to the US developed (Fig. 9E). After the second injection of gabazine the excitatory response to the US disappeared and the CR recovery was accompanied by the restoration of neuronal activity (Figs. 9B, C, and F). Toward the end of the session the baseline activity even exceeded its pre-injection level. (Fig. 9C, F).

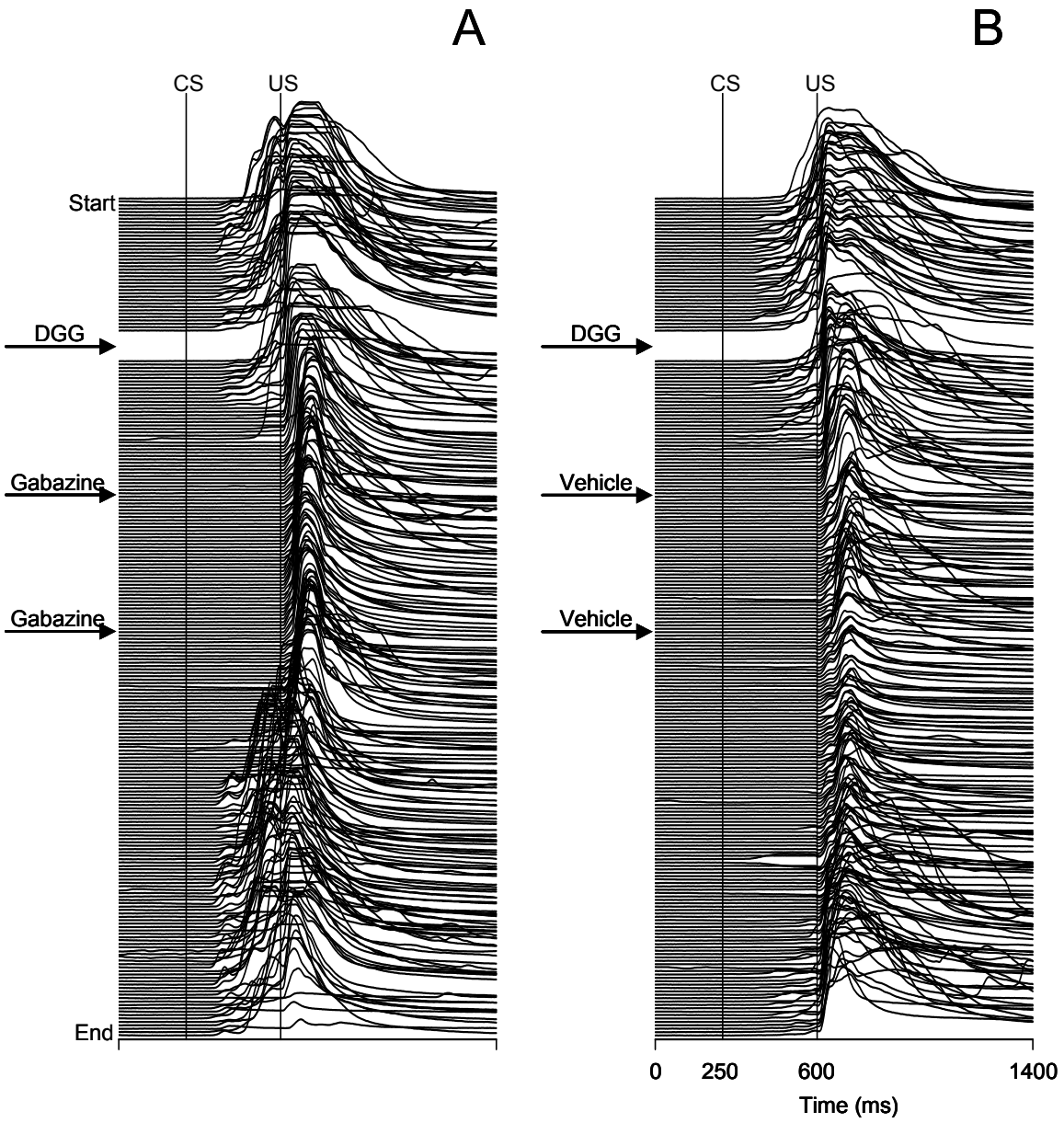
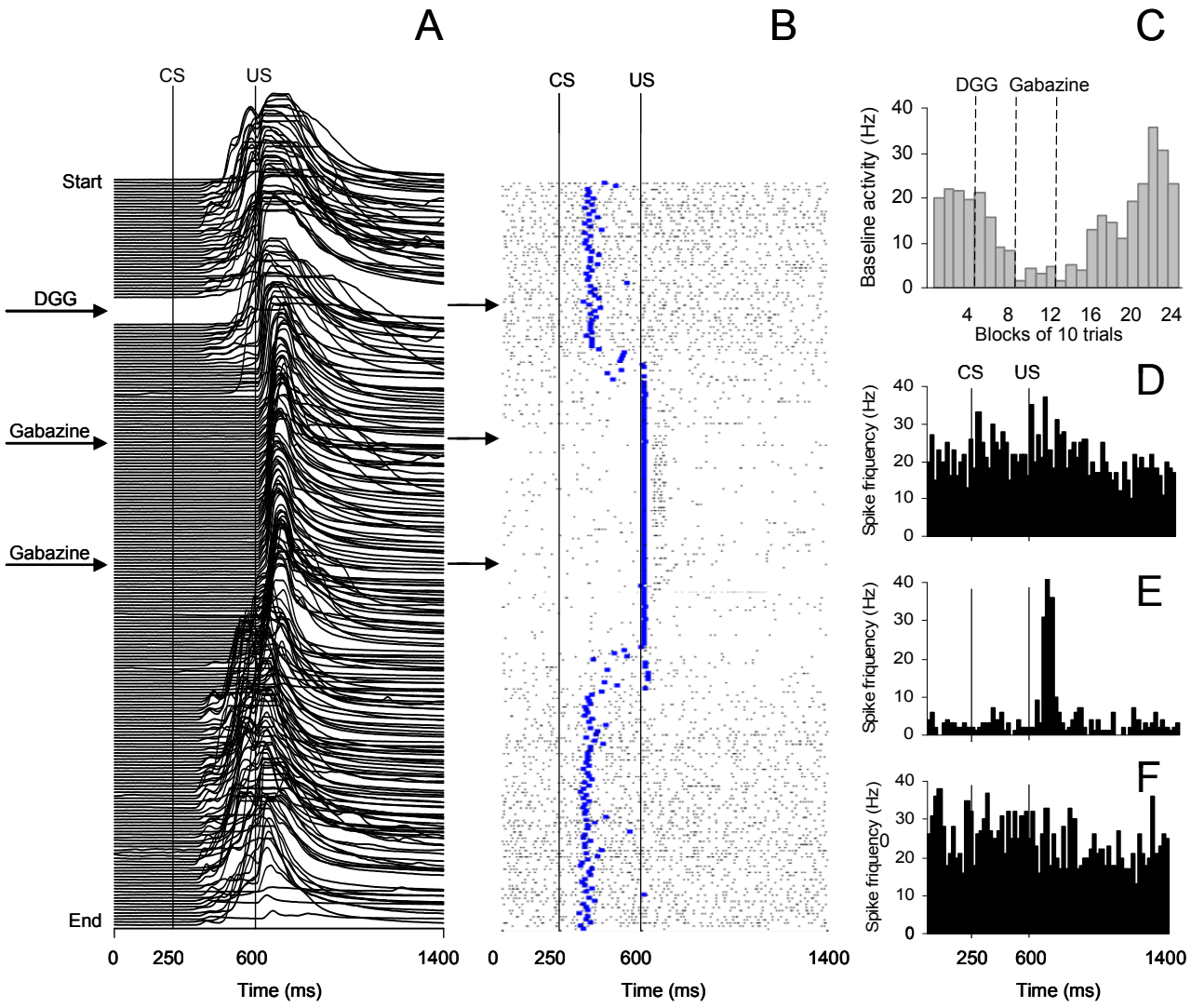


Fig. 8. Individual examples of the behavioral effects of sequential injections of DGG and gabazine compared to the control injections of DGG and vehicle (aCSF) into the IO of the same subject. *A*: After the DGG injection into the IO, CRs were abolished within 7 minutes of resuming post-injection training. Twenty minutes after the first injection of gabazine, CRs recovered with some shortening of CR latency. Their amplitude and shape were comparable to pre-injection CRs. *B*: In the control experiment, DGG again abolished CRs within 7 minutes. Injecting vehicle (aCSF) in the same manner and time frame as gabazine in the previous experiment resulted in CRs appearing much later with latencies longer and amplitudes smaller than pre-injection CRs.

Fig. 9. An example of the parallel effects of sequential injections of DGG and gabazine on CR performance and on the activity of an IN cell. This experiment consisted of 240 trials with the injection of DGG administered after 40 pre-injection trials and those of gabazine administered at trials 80 and 120. *A*: Stack plot of all eyeblinks showing CRs gradually being abolished after DGG and then completely restored about 15 minutes after the second gabazine injection. *B*: Raster plot of IN cell activity during the same experiment. The experiment starts at the top with each row representing one trial. Small dots mark the occurrence of action potentials and large dots denote the onset of eyeblinks. Coinciding with the timing of the behavioral CR abolition, the firing rate of this cell's activity was severely suppressed. The recovery of neuronal activity occurred after the second gabazine injection and it paralleled the behavioral recovery of CRs. *C*: Bar plot representing changes in baseline activity during the experiment. *D*: Peri-stimulus histogram of the same IN unit constructed for 40 trials before the injection. *E*: Peri-stimulus histogram of the same cell during 40 post-DGG injection trials when behavioral CRs were abolished and cell activity was suppressed. *F*: Peri-stimulus histogram constructed for 40 post-gabazine trials when complete recovery of behavior and cell activity was observed. Bin width for histograms in *D, E,* and *F* is 20 ms. CS, onset of conditioned stimulus; US, onset of unconditioned stimulus.

Fig. 9.


A
B
C
CS
US
Start
DGG
Gabazine
Gabazine
End
0
250
600
1400
Time (ms)
DGG
Gabazine
Baseline activity (Hz)
Blocks of 10 trials
D
E
F
Spike frequency (Hz)

4.5 Discussion and Conclusions

The data presented here demonstrate that blocking the inhibition to the IO by the GABA-A receptor antagonist gabazine shortens CR latency and increases neuronal activity in the IN. The most important finding was that the recovery of DGG-suppressed CRs and IN neuronal activity can be significantly accelerated by intra-olivary injections of gabazine. We also found that systemic effects of harmaline on CR expression are not mediated by the eyeblink-related parts of the IO.

4.5.1 Effect of harmaline on CR expression

Harmaline is an alkaloid which has been known since 1894 to produce a high frequency tremor in mammals (Llinas and Volkind, 1973; Headley et al., 1976). Systemic administration of harmaline produces an 8-12 Hz body tremor in decerebrated cats (Lamarre and Mercier, 1971; Lamarre and Weiss, 1973). Single-unit recordings after systemic administration of harmaline revealed Purkinje cells to be rhythmically activated at 8-12 Hz via climbing fiber afferents from the inferior olive (De Montigny and Lamarre, 1973; Llinas and Volkind, 1973). Direct electrophysiological recordings from the IO confirmed these results and detected rhythmic activity only in caudal parts of the medial and dorsal accessory inferior olive, not in its rostral half (De Montigny and Lamarre, 1973; De Montigny and Lamarre, 1975). The mechanism through which harmaline affect the IO is not known.

Systemic harmaline disrupts acquisition of conditioned nictitating membrane responses in the rabbit, but it does not prevent subsequent acquisition of conditioning or performance of unconditioned responses (Turker and Miles, 1984; Harvey and Romano,

1993). Importantly, systemic harmaline also reduces the incidence of previously acquired CRs (Turker and Miles, 1984). Our data confirm the latter finding. Approximately ten minutes after we injected harmaline, CRs were mostly suppressed and rabbits developed body/neck tremor although eyelid movements were not affected by the harmaline-induced tremor.

While our experiments with systemic administration of harmaline were geared toward reexamining previous studies, our testing the effect of harmaline injections in the IO on CR expression were novel experiments. These experiments offer surprising insights in understanding the mechanisms of harmaline action in the rabbit. We found that intra-olivary application of excess harmaline, at sites where previous application of DGG abolished CRs, had no effect on CRs. These finding extend previous reports of the rostro-caudal gradient of IO sensitivity to harmaline (De Montigny and Lamarre, 1973; De Montigny and Lamarre, 1975), and it suggests that the eyeblink-related rostro-medial part of the IO is insensitive to harmaline. We conclude that the systemic effects of harmaline on CR performance are most likely not mediated by direct effects of this drug on IO eyelid representation.

4.5.2 Effect of gabazine on CR expression and on IN neuronal activity

Since intra-olivary injections of harmaline most likely did not increase IO tonic activity, a second drug candidate, gabazine was tested. As a GABA-A receptor antagonist (Ueno et al., 1997), gabazine was chosen to increase tonic activity in the IO because it is known that the IO receives GABA-ergic afferent projections (Fredette and Mugnaini, 1991; Ruigrok and Voogd, 1995; Svensson et al., 2005; Bengtsson et al., 2004).

We discovered that activating the IO with gabazine does not affect CR incidence but instead shortens CR latency and increases CR amplitude. This finding is remarkably similar to the phenomenon of short-latency CRs reported in experiments blocking the inhibition directly in the IN (Ohyama et al., 2003; Ohyama et al., 2006). Since our electrophysiological recordings revealed that blocking IO inhibition increased neuronal activity in the IN, we propose that a similar neuronal mechanism underlies the shortening of CR latency both after disinhibiting the IO and the IN. Specifically, activating the IO with gabazine most likely suppresses simple spikes in Purkinje cells resulting in decreased IN inhibition. This indirect activation of the IN should yield a similar behavioral outcome as the direct pharmacological disinhibition of the IN. It should be noted that an IO gabazine-induced disinhibition of the IN is relatively mild since a complete block of GABA-A receptors or of chloride channels in the IN leads to CR abolition (Aksenov et al., 2004).

4.5.3 Effects of a combined block of glutamate and GABA-A receptors

The purpose of these experiments was to examine changes in CR expression and in cerebellar nuclear activity associated with blocking glutamatergic sensory inputs to the IO under conditions where the tonic effects of DGG were opposed by gabazine. The results of this preliminary study are highly encouraging. We found that gabazine significantly accelerated the recovery of CRs abolished by the prior injection of DGG. Interestingly, the recovered CRs did not subsequently extinguish (as the cerebellar learning hypothesis would predict) - they persisted through the end of the experiments. The behavioral data were well complemented by recordings of IN activity showing that the CR recovery was paralleled by the recovery of the IN's spontaneous firing rate. These findings are inconsistent with the

cerebellar learning hypothesis and suggest that the IO's US signals are most likely not required for CR retention. Since the IO receives inhibitory inputs from cerebellar and extra-cerebellar sources (Hesslow and Ivarsson, 1996; Gibson et al., 2002), it is possible that the main IO function could be the setting of functional tonic states of the cerebellum during learning.

We would like to emphasize that the above conclusion is preliminary not only because of the insufficient sample size, but also because of our concerns with the dynamics of the gabazine-induced CR recovery. Specifically, the recovery of neuronal activity in the IN and of behavioral CRs was delayed when compared to injections of gabazine-alone. We can not exclude the possibility that some glutamate receptors were already active by the time of CR recovery. If so, then these functioning receptors may have been sufficient in transmitting sensory information to the cerebellum, thus preventing CR extinction. This seems unlikely because in control experiments, where the same amount of glutamate antagonist and vehicle were injected, CR recovery occurred much later than in DGG-gabazine experiments. Nevertheless, addressing this question satisfactorily will require direct electrophysiological measurements of IO responses to the US at all stages of the combined DGG/gabazine experiment.

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CHAPTER 5. GENERAL CONCLUSIONS

5.1 General conclusions

We have investigated the role of the IO in the in the expression and retention of classically conditioned eyeblinks in the rabbit. The main goal of this dissertation was examining one of the central tenets of the cerebellar learning hypothesis – the presumed teaching role of the IO in maintaining cerebellar traces of memory. In the main part of the dissertation we focused on determining whether inactivating the IO or blocking the glutamatergic sensory inputs in the IO could be used for testing the cerebellar learning hypothesis. In these experiments we were the first to show that inactivating the IO or blocking glutamate receptors in the IO abolishes CRs not because of unlearning, but because these interventions produce tonic dysfunction of cerebellar circuits. These fundamental findings disprove established interpretations of IO manipulation experiments and weaken the evidence for the cerebellar learning hypothesis. In the second part of the dissertation, we initiated studies with an objective to develop a principally new pharmacological protocol that would block IO sensory inputs without producing cerebellar tonic dysfunction. In these studies we found that the physiological effects of glutamate blockers could be potentially offset by the GABA-A antagonist gabazine. This finding opens a new and very promising route for testing IO function.

The specific major findings of our research have already been deliberated upon in the discussion section at the end of each chapter. In summary, the experiments described in this dissertation clearly demonstrated that:

1. Inactivating the DAO with muscimol or blocking both AMPA and NMDA receptors in the DAO abolishes CRs on a time course most likely determined by the dynamics of drug diffusion, not repeated presentations of the CS-US.
2. Blocking glutamate neurotransmission in the DAO induces tonic dysfunction in cerebellar circuits as evidenced by our single-unit recordings at the cerebellar output in the IN. Cerebellar dysfunction and not extinction is responsible for the CR suppression.
3. Selectively blocking AMPA/kainate receptors in the DAO abolishes the expression of well-learned eyeblink CRs in the rabbit. Again, the gradual disappearance of CRs was a time-dependent function of diffusion rather than post-injection training.
4. Selectively blocking AMPA/kainate receptors in the DAO decreases tonic activity in the IN and alters its neuronal responses to paired stimuli. Changes in IN baseline activity were not stimulus-dependent.
5. Conclusions 1-4 collectively indicate that inactivating the IO or blocking its glutamatergic sensory inputs does not induce unlearning as previously thought. We have demonstrated that all these treatments suppress CR performance by producing tonic dysfunction of cerebellar circuits, and not by a US-blocked unlearning of conditioned responses.
6. The CR suppression that follows systemic administration of harmaline is not mediated by the IO as evidenced by the absence of any harmaline effects when injected at sites where DGG reliably abolished CRs. The DAO seems to be

harmaline-insensitive, therefore, this drug is not suitable for offsetting tonic effects of IO glutamate blockers.

7. Blocking GABA-A receptors in the IO shortened CR latency, produced tonic eyelid closure and increased the spontaneous firing rate of IN cells. This finding indicated that gabazine could be potentially used to offset the tonic effect of IO glutamate blockers.
8. Sequential injections of DGG and gabazine demonstrated that the recovery of CRs and of IN neuronal activity can be significantly accelerated by gabazine. In addition, the recovered CRs did not extinguish. These preliminary findings emphasize the importance of a near normal rate of IO neuronal firing for the performance of CRs, and that maintaining their excitability at near normal levels is more important for the generation of CRs than is their modulation by olivary inputs.

5.2 Recommendations for future research

The fundamental contribution of the presented research is that it explained that IO inactivation-induced CR deficits are a result of a learning-independent physiological mechanism that interferes with tests of possible IO learning functions. Importantly, we believe that we found a new technique which could neutralize the tonic interference of IO manipulations. Since the combined DGG and gabazine method has extremely promising uses and implications, our discussion of future research will focus on issues related to its verification and exciting applications.

First of all, since we have presented only exploratory electrophysiological recordings of IN neuronal activity in combination DGG-gabazine experiments, further tests will be necessary to confirm our findings in a more representative sample of animals and IN neurons. Assuming the confirmation of our findings, additional tests will have to be conducted to confirm that no US signals are conveyed to the cerebellum during the gabazine induced CR recovery. Confirming this expected result would offer a principally new and definitive method for examining the IO contribution not only to the retention of CRs, but also to learning new CRs in naïve animals. Alternatively, if the current DGG/gabazine approach will prove to work only in conditions of incomplete glutamate receptor block, new types of drug combinations, such as NBQX and picrotoxin could be investigated for their potential to separate the tonic and learning functions of the IO.

Although it was not the main subject of our investigations, our research offered one of the best illustrations of the IO regulating tonic activity in cerebellar circuits. Our data questions the physiological significance of this process. Does the IO set a specific level of

cerebellar activity during certain motor and learning tasks? Long term recordings from the same set of cerebellar neurons during a variety of behavioral states and learning situations could offer important insights into this fundamental question.

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